Molecular Cloning and Characterization of Anaerobiosis-inducible Promoters from *Escherichia coli* and *Salmonella typhimurium*

BY

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ABSTRACT

Salmonella typhimurium and *Escherichia. coli* are facultative anaerobes. They can grow in the presence of oxygen or in the absent of oxygen. A set of genes are induced under anaerobic growth conditions. How these genes are regulated at the molecular level is still not clearly defined. The aim of this thesis was to clone and to determine the sequences of some anaerobiosis-inducible promoters from *S. typhimurium* and *E. coli*, with emphasis on that of *S. typhimurium*.

In this study, several anaerobiosis-inducible promoters were cloned from both *S. typhimurium* and *E. coli*. But only 4 promoters from *S. typhimurium* and 1 promoter from *E. coli* were sequenced. The sequences of these promoters were compared for homology among themselves and with other 13 known promoter sequences of anaerobiosis-inducible genes. An anaerobiosis-sense element (ASE) with consensus sequence AATT---ATTTAAT--AT was deduced from the homology search.

To begin the cloning of anaerobiosis-inducible promoters from *S. typhimurium*, promoter-probe plasmid pKK232.8 (Brosius, 1984) was employed. pKK232.8 plasmid vector contains a promoterless chloramphenicol acetyl transferase gene (*cat*) as the "reporter" gene. When a promoter-containing DNA fragment was inserted into the cloning site of the vector pKK232.8, the bacteria harboring this recombinant plasmid became chloramphenicol resistant.

Chromosomal DNA prepared from S. typhimurium LT2 was subjected to restriction enzyme Sau3A partial digestion and was ligated to restriction enzyme BamH1 complete digested pKK232.8 plasmid. This recombinant plasmids were used to transform bacterial strain JM107 or MC1061-5. Ampicillin resistant transformants were selected aerobically and replicated to chloramphenicol containing agar plates which were incubated anaerobically. Anaerobic chloramphenicol resistant colonies were than picked and patched onto aerobic and anaerobic incubated chloramphenicol plates. Isolates showed better anaerobic growth were further analysis by CAT assay. Six anaerobiosis-inducible promoters were then isolated from S. typhimurium. Two of promoters (in pHSK1 and pHSK8) with higher induction ratio (anaerobic activity/aerobic activity) were sequenced and futher studied. The anaerobic expression of promoter in pHSK8 was further increased with the addition of nitrate under anaerobic condition. oxrA was shown to be required for the nitrate induction of the promoter in pHSK8.

Another promoter-probe plasmid pFZY1, constructed by Koop et al. (1987) were used for the isolation of anaerobiosis-inducible promoters at later stage when Dr. Bourgeois kindly gave me this plasmid. This plasmid contains a promoterless lacZ gene as the "reporter" gene. With this promoter-probe plasmid, several anaerobiosis-inducible promoter-containing DNA fragment were isolated from E. coli, and two of them have induction ratio greater

than 8. Incidentally, they both contained the promoter of glpT (coding for glycerol-3-phosphate permease) but with different length of coding regions. Besides anaerobiosis-induction, the expression of glpT was also increased by glycerol-3-phosphate under aerobic condition, but its expression was decreased by nitrate and glucose under anaerobic condition. Anaerobic expression of glpT was also shown to be fnr-dependent.

With the promoter-probe plasmid pFZY1, two more anaerobiosis-inducible promoters from S. typhimurium were cloned and sequenced. One of the promoters was inducible by nitrate in addition to anaerobiosis. The expression of the promoters were tested in S. typhimurium strain JR501 and that with oxrA mutation. It was demonstrated that oxrA mutation was not required for anaerobic induction for these promoters.

To sum up, several anaerobiosis-inducible promoters were successfully cloned from E. coli and S. typhimurium using promoter-probe plasmids. Five of the cloned promoters with higher anaerobic induction ratio were sequenced and characterized. These promoters had different regulation. They showed different responds to fnr mutation or the presence of nitrate. A putative anaerobiosis-sense element (ASE) was suggested.

Statement

All the experimental works reported in this thesis were performed by the author, unless specifically stated otherwise in the text.

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Abbreviations

Abbreviations used in this thesis without definition include:

EDTA:	ethylenediaminetetraacetic acid
IPTG:	isopropyl-1-thio-ß-galactoside
Kb:	kilo-bases
MOPS:	morpholinepropanesulfornic acid
dNTPs:	deoxyribonucleotides (dATP, dTTP, dCTP, dGTP)
ONPG:	orthonitrophenyl-ß-D-galactoside
PEG:	polyethylene glycol
RF:	replicative form
SDS:	sodium dodcyl sulfate
TEMED:	N,N,N',N'-tetramethyl-ethylenediamine
Tris:	tris(hydroxymethyl)aminomethane
Xgal:	5-Bromo-4-chloro-3-indolyl-ß-D-galactoside

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Chapter 1.

Introduction and Literature Review

I. INTRODUCTION

A. General Introduction

This thesis is the report of my research on the molecular cloning and characterization of anaerobiosis-inducible promoters from the facultative bacteria - Escherichia coli K12 (Bachmann, 1983) and Salmonella typhimurium LT2 (Sanderson and Roth, 1988). My major effort was concentrated on that of S. typhimurium.

After reviewing relevant literatures in this chapter, the next chapter will describe the "shot gun" approach which was used for the hunting of anaerobiosis-inducible promoter containing DNA fragments. Two promoter probe plasmids pKK232.8 (Brosius, 1984) and pFZY1 (Koop et al., 1987) used in this study will be described. Several anaerobiosis-inducible promoter containing DNA fragments were cloned from both S. typhimurium and E. coli, but only 4 clones from S. typhimurium and 1 clone from E. coli were sequenced and characterized. The subcloning and sequencing strategy are described in chapter 3. The gene expression of the promoters are discussed in chapter 4. The last chapter is "General Discussion".

Using promoter probe plasmid pKK232.8, six putative anaerobiosis-inducible promoter clones were found. Among this six putative anaerobiosis-inducible promoters, two

promoters (pHSK1 and pHSK8) with anaerobic induction greater than 5-fold were further analysed. A 1.3 kb DNA fragment from pHSK1, which contained the anaerobiosis-inducible promoter was subcloned and sequenced. A 2.0 kb fragment from pHSK8 was subcloned and sequenced.

Since pKK232.8 is a multi-copy-number plasmid in the bacteria, a high background chloramphenicol resistance may occur aerobically when the promoter is expressed weekly. Thus it is difficult to detect anaerobiosis-inducible promoters with a basal aerobic expression level. Hence, a low-copy-number vector was used to overcome this problem. The promoter probe plasmid pFZY1, a gift from Dr. S. Bourgeois (Koop et al., 1987) was used for isolation of anaerobic inducible promoters. pFZY1 is a low-copy-number plasmid utilizing a promoterless *lacZ* gene as the "reporter" gene. With this plasmid, anaerobiosis-inducible promoters were comparatively easier to isolate. The advantages of pFZY1 plasmid are its low-copy-number property and the simplicity of the ß-galactosidase enzyme assay. With this promoter probe plasmid, promoters were cloned from both *E. coli* and *S. typhimurium*.

Ninety-two anaerobically expressed promoters were screened for anaerobiosis induction. Two strong anaerobiosis-inducible promoters containing DNA fragments (1.4 kb and 3.6 kb) were isolated from *E. coli*. These two fragments both contained the same promoter of *glpT* (glycerol-3-phosphate permease gene) with different

lengths of coding region. The 1.4 kb fragment contained both the regulatory region of glpT and glpA. Catabolite repression was significant under anaerobic condition. This observation indicated that catabolite repression may somehow interact with anaerobiosis induction for glpT. Moreover, since both glpAB and glpT were induced by anaerobiosis. It is speculated that a common regulatory region in the promoter may be responsible for the anaerobiosis induction of glpT and glpAB.

Similarly, a promoter library was constructed for S. typhimurium with small sau3A fragments (about 0.5 kb). Sixty-five clones were screened for anaerobiosis induction of β-galactosidase activities and 3 putative anaerobiosis-inducible promoter containing DNA fragments were isolated. To facilitate the analysis of the cloned DNA fragments, the sequences on both sides of the multiple-cloning sites of pFZY1 was determined. Two oligonucleotide primers (20-mers) were synthesized with oligonucleotide synthesizer (Applied Biosystem). With the oligonucleotide primers, large amount of the anaerobiosis-inducible promoter containing DNA fragments were amplified from small amount plasmid by PCR. The PCR fragments were subcloned and sequenced.

To conclude, one anaerobiosis-inducible promoter was cloned and sequenced from E. coli and identified to be the promoter of glpT. Four anaerobiosis-inducible promoters were cloned and sequenced from S. typhimurium. In addition

to induction by anaerobiosis, two cloned promoters from S. typhimurium were also induced by nitrate under anaerobic condition. The property of nitrate induction under anaerobic condition for one of the promoters was shown to be oxrA dependent. Putative FNR(OxrA) binding site was suggested for the fnr(oxrA) dependent promoters isolated when the sequences were searched for homology with the consensus FNR binding site sequence in E. coli (Eiglmeier et al., 1989). Similarly, NarL binding sites were suggested for the promoters regulated by nitrate. When the promoters sequences in this study were aligned with all known anaerobiosis inducible promoter sequences, a putative consensus sequence which responses to anaerobiosis was suggested.

B. Purpose of study

I am interested in understanding the genetic regulation of aerobic-anaerobic shift of the facultative Enterobacteriacea. S. typhimurium and E. coli are good model systems for the study.

Regulation of gene expression generally involves three elements: a signal effector, a regulatory protein, and a sequence within the promoter region of a gene. To understand the genetic regulation at the molecular level, one have to identify the DNA sequence in the promoter region of the gene, which is recognized by the regulatory protein. This is usually done by determining and comparing DNA sequences of promoters of the genes which are regulated by the same signal. At the beginning of this research, only a few anaerobic genes have been cloned and sequenced. Comparison of only a few genes was not enough for the identification of important regulatory sequences. Thus, the purpose of this study is to clone and to determine the sequences of promoters of anaerobiosis-inducible genes. With more anaerobiosis-inducible promoter sequences known, we may be able to identify their consensus regulatory sequence which is the target sequence for anaerobiosis induction.

II. LITERATURE REVIEW

A. Global control of aerobic-anaerobic shift

S. typhimurium and E. coli are facultative bacteria. They can grow either in the presence or absence of oxygen. However, the bacteria exhibits distinct growth pattern when shift from aerobic growth to anaerobic growth. The notable changes are in the routes of carbon source utilization and energy metabolism (Reichelt and Doelle, 1971; Thomas et al., 1972). During aerobic growth, oxygen serves as the terminal electron acceptor for a respiratory chain that provides both a hydrogen sink for metabolically driven electrons and the means for oxidative energy production. A set of proteins were induced by aerobiosis (Smith and Neidhardt, 1983b). Actually this set of proteins were repressed by a repressor encoded by arcA gene under anaerobic condition (Iuchi and Lin, 1988). On the other hand, in the absence of molecular oxygen, the bacteria can use other electron acceptors such as nitrate, nitrite, thiosulfate, or trimetylamine-N-oxide. When electron acceptors are all absent, the bacteria carry out mixed-acid fermentation metabolic pathway, by which pyruvate undergoes stepwise reduction to a variety of end products and ATP is produced exclusively by substrate-level phosphorylations. Formate, acetate, ethanol, succinate, lactate, H_2 and CO_2 are the end products.

The molecular response to anaerobiosis is at the

whole-cell level, under a global control (Gottesman, 1984). Smith and Neidhardt (1983a) demonstrated with two dimensional gel electrophoresis analysis that 18 proteins increased two- to eleven-fold after a shift from aerobic to anaerobic growth conditions. These 18 polypeptides include at least four glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, fructose-6-phosphate kinase, pyruvate kinase, and enolase) and pyruvate formate lyase. To study the global control in S. typhimurium, Spector et al. (1986) also demonstrated with two dimensional gel electrophoresis that at least 30 proteins were induced under anaerobiosis. They showed that some of the anaerobiosis inducible proteins were also induced by starvation or heat shock. An overlap between osmotic and anaerobic stress responses was demonstrated in the gene expression study of several genes in response to osmotic and anaerobic stress. The osmotically induced proU and ompC genes require anaerobic growth for optimum induction while the anaerobically induced tppB gene is also regulated by osmolarity (Bhriain et al., 1989).

Besides the study on the global changes in protein pattern during aerobic-anaerobic shift with two dimensional gel electrophoresis, lac-operon fusion technique was also used to identify an oxygen-regulated stimulon of S. typhimurium at the transcriptional level (Aliabadi et al., 1986). A collection of anaerobically induced gene fusions of E. coli were also isolated and their map locations on the chromosome were identified. The

anaerobically induced fusions were scattered around the E. coli chromosome more or less at randomly (Winkelman and Clark, 1986). Using the gene fusions technique, Clark (1984) estimated that the number of anaerobically induced genes in E. coli was about 50.

B. Identified anaerobiosis-inducible genes

So far, about 40 anaerobiosis inducible genes have identified and about 15 genes have been sequenced. The indentified number is close to that suggested by various studies (Smith and Neidhardt, 1983a; Spector et al., 1986; Clark, 1984). They are listed in alphabetical order in table 1.

Table 1. Genes which are shown to be anaerobiosis inducible in *Salmonella typhimurium* (ST) and/or *Escherichia coli* (EC).

Gene	Level of study[a]			Regulation[b]		Reference
	Enzyme	*lac*-fusion	Promoter sequence	*fnr*	others[c]	
acd (acetaldehyde coenzyme A dehydrogenase)	EC					Lorowitz and Clark, 1982
ackA (acetate kinase)	EC, ST	ST	EC			Matsuyaam et al., 1989 Kwan et al., 1988 Kwan (unpublished result) Smith and Neidhardt, 1983a
adh (alcohol dehydrogenase)	EC		EC		nitrate(-)	Wong and Barrett, 1984 Goodlove et al., 1989 Lorowitz and Clark, 1982
aid (alkylation-inducible)		EC				Volkert et al., 1989
ant (anaerobic electron transport)		EC				Yerkes et al., 1984
ansB (L-asparaginase II)	EC	EC	EC	-		Jennings and Beacham, 1990 Jerlstrøm et al., 1987
aspA (aspartase)	EC	EC		-		Woods and Guest, 1987 Jerlstrøm et al., 1987
bet (osmotic induction)		EC				Eschoo, 1988

Table 1 (cont'd)

cadA (lysine decarboxylase)	EC			0	nitrate(0)	Auger and Bennett, 1989
cob (cobalamine biosynthesis)		ST				Jeter and Roth, 1987
dmsABC (dimethyl sulfoxide reductase)	EC	EC	EC	-	nitrate(-)	Cotter and Gunsalus, 1989 Bilous and Weiner, 1985 Bilous et al., 1988
eno (enolase)	EC					Smith and Neidhart, 1983a
fdhF (formate dehydrogenase H)	EC,ST	ST, EC	EC	0[d]	ntrA(-) gyrA(+) oxrC(-) nitrate(-)	Barrett et al., 1984 Birkmann et al., 1987 Axley and Stadtman, 1988 Zinoni et al., 1986 Stewart and Parales, 1988
fdn (formate dehydrogenase N)	ST, EC			-[e]		Paveglio et al., 1988 Lambden and Guest, 1976 Birkmann et al., 1987
frdABCD (fumarate reductase)	EC,ST	EC	EC	-	nitrate(-)	Iuchi et al., 1986 Cole, 1982 Jones and Gunsalus, 1987 Lambden and Guest, 1976 Jameison et al., 1986 Chan, 1987
fumB (fumarase)	EC	EC	EC	-		Woods and Guest, 1987 Bell et al., 1989b

Table 1 (cont'd)

gap (D-glyceraldehyde-3-phosphate dehydrogenase)	EC		EC			Smith and Neidhart, 1983a Branlant and Branlant, 1985
glpABC (glycerol-3-phosphate dehydrogenase)	EC	EC	EC	-		Kuritzkes et al., 1984 Cole et al., 1988 Iuchi et al., 1985
glpT (glycerol-3-phosphate permease)		EC	EC	-	nitrate(-)	This study Eiglmeier et al., 1987
hyd (hydrogenase)	EC,ST	ST		0	oxrC(-)	Barrett et al., 1984 Jamieson and Higgins, 1985 Zinoni et al, 1984 Sawers et al., 1985
hyd-17 (hydrogenase)		EC		0[d]	nitrate(-)	Birkmann et al., 1987
hydC (hydrogenase)		EC		-		Wu and Mandrand-Berthelot, 1986
ilv (isoleucine biosynthesis)		EC				Reams et al., 1987
leu (leucine biosynthesis)		EC				Reams et al., 1987
men (menaquinone)		ST				Kwan and Barrett, 1983
nad (NAD biosynthesis)		ST				Holley et al., 1985

Table 1 (cont'd)

narGHJI (nitrate reductase)	EC,ST	ST,EC	EC	-	nitrate(+)	Blasco et al., 1989 Chippaux et al., 1981 Kwan and Wong, 1986 Showe and DeMoss, 1968 Chan, 1987
narK (nitrate transport)		EC		-	nitrate(+)	Stewart and Parales, 1988 Noji et al., 1989
narX (Nitrate sensor)		EC			nitrate(+)	Stewart and Parales, 1988 Nohno et al., 1989
nirB (nitrite reductase)	EC	EC	EC	-		Griffiths and Cole, 1987 Jayaraman et al., 1987 Newman and Cole, 1978
pepN (aminopeptidase N)		EC	EC			Bally et al., 1986 Foglino et al., 1986
pepT (peptidase T)		ST		-	oxrC(-)	Strauch et al., 1985
pfl (pyruvate formate lyase)	EC	ST,EC	EC	-	pflR(-)	Wong et al., 1989 Wong and Kwan, 1988 Sawers and Bθck, 1988 Rodel et al, 1988 Pecher et al, 1982
pfkA (phophofructase kinase)	EC		EC			Smith and Neidhart, 1983a Hellinga and Evans, 1985
pgk (phosphoglucose kinase)	EC					Nellemann et al., 1989

Table 1 (cont'd)

phs (sulfite reductase)	ST	ST			Hallenbeck et al., 1989 Clark and Barrett, 1987
pta (phosphotransacetylase)	ST	ST			Kwan (unpublished result)
sodA (pro Mn-superoxide dismutase)	EC			nitrate(+)	Privalle et al., 1989
torA (Trimethylamine N-oxide reductase)	ST	EC	0	nitrate(-)	Pascal et al., 1984 Kwan and Barrett, 1983
thi (thiamine biosynthesis)		EC			Reams et al., 1987
tppB (tripeptide permease)		ST	0	oxrC(-) ompR(-) envZ(-)	Jamieson and Higgins, 1984 Gibson et al, 1987
tyr (tyrosine biosnthesis)		EC			Reams et al., 1987

a. Anaerobic induction was demonstrated at the level of enzyme synthesis (by enzyme assay or two dimensional gel electrophoresis) ; Anaerobic induction was demonstrated at the level of gene expression (by β-galactosidase enzyme assay of lac-fusions).
b. 0, no effect (activity not change in fnr background); -, negative effect (reduced expression fnr background). Those not mentioned are not tested.
c. +, positive effect (increased expression); -, negative effect (decreased expression); 0, no effect in the present of the indicated mutations or nitrate.
d. After growth in the presence of formate.
e. After growth in the presence of nitrate.

Their induction (derepression) by anaerobiosis was mainly demonstrated by two dimensional gel electrophoresis and gene fusion methods. With two dimensional gel electrophoresis, the amount of proteins synthesized (enzyme synthesis) under aerobic and anaerobic conditions were determined. However, whether the anaerobic induction is at the translational level or transcriptional level is not known. On the other hand, gene fusion (transcriptional fusion) showed that the induction was at the transcriptional level. All the gene listed in Table 1 have been studied with lac operon fusion technique except fdn, cadA, sodA, sulfite reductase.

The genes of Table 1 can be roughly classified into the following categories:

1. genes involved in the glycolytic pathways;
2. genes involved in mixed-acid fermentative pathway;
3. genes involved in anaerobic respiratory pathways;
4. genes involved in biosynthesis (cobalamine, NAD, thiamine, leucine, isoleucine, tyrosine);
5. genes involved in catabolism (utilization of peptides,aspartate, glycerol-3-phosphate);
6. genes responds to environmental stress (osmotic stress).

C. Genetics of anaerobic regulation

i. Redox control

Nitrate and fumarate can act as electron acceptors in the absence of oxygen. Oxygen, nitrate and fumarate are of different redox potentials. The expression of hydrogenase (hyd) and formate dehydrogenase (fdh) in E. coli was repressed by oxygen, and repressed by nitrate under anaerobic condition. However, fumarate only partially repressed their expression (Pecher et al., 1983). Pecher et al. (1983) suggested that the repressive effect of a terminal electron acceptor depends on the level of its redox potential. Unden and Guest (1984) speculated that the regulatory FNR protein (to be discussed in following paragraphs) may bind to an unidentified effector or effectors which signal the redox state of the cell. On the other hand, FNR protein itself could be redox-sensitive.

ii. DNA conformation

It has been generally accepted that the conformational change of DNA is an important factor for the regulation of gene expression. The expression of some genes is known to be affected by DNA supercoiling which is controlled by the activity of topoisomerase I and gyrase (Fisher, 1984). Strict aerobic mutants and strict anaerobic mutants were isolated from S. typhimurium (Yamamoto and Droffner, 1985). Strict

aerobic mutants contained a defective DNA gyrase subunit A gene (gyrA), while strict anaerobic mutants contained a defective DNA topoisomerase I gene (topI). The results led to the conclusion that activity of topoisomerase I, associated with relaxation of chromosomal DNA, is necessary for the expression of genes required for aerobic growth, where activity of gyrase, associated with supercoiling of chromosomal DNA, is necessary for the expression of genes required for anaerobic growth. However, anaerobic induction of E. coli formate dehydrogenase (hydrogenase-linked) is enhanced by gyrase inactivation (Axley and Stadtman, 1988).

iii. fnr(oxrA) regulatory gene

The precise signal the bacterial cells uses to recognize an oxygen deficiency is still not clear. One protein regulator of the system has been identified as the product of the fnr (nirR, nirA) gene (Shaw and Guest, 1981), a positive regulatory protein with much homology with the CRP protein (Shaw et al., 1983). Mutations in fnr are recessive, and fnr mutants are unable to express the systems of the anaerobic respiration with fumarate, nitrate ,and nitrite (Lambden and Guest, 1976; Newman and Cole, 1978; Chippaux et al., 1981). With regard to S. typhimurium, Strauch et al. (1985) identified two regulatory loci called oxrA and oxrB. The map location of oxrA and the complementation by cloned fnr from E. coli indicated that oxrA is equivalent to fnr (Jamieson and Higgins, 1984). The effect of fnr (oxrA)

mutations on enzyme synthesis and gene expression in E. coli and S. typhimurium has been reviewed by Stewart (1988). Not all anaerobiosis-inducible genes are regulated by fnr (oxrA) (Aliabadi et al., 1986; Jamieson and Higgins, 1986). Jamieson and Higgins (1986) suggested that there were two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in S. typhimurium - fnr-dependent or oxrC-dependent. The primary defect of oxrC mutation is a deficiency in phosphoglucose isomerase activity, implying that a product of glycolysis functioned as an anaerobic regulatory signal. The fnr-dependent genes encode proteins for primarily respiratory functions, whereas the oxrC-dependent genes serve fermentative or biosynthetic roles. The significance of fnr protein on cellular protein composition of E. coli was studied by Sawers and Böck (1988). Analysis of the cellular proteins of fnr mutant and wild type of E coli with two dimensional electrophoresis showed that the expression of 21 anaerobically inducible polypeptides are independent of the presence of FNR protein. On the other hand, 22 anaerobically inducible polypeptides were shown to be reduced in a fnr mutant. A total of 8 proteins were shown to be reduced in a fnr mutant only in aerobically grown cells indicating that FNR protein has a function in the presence of oxygen (Sawers and Böck, 1988).

From the study of a translational fusion of fnr to the gene for ß-galactosidase (lacZ), it has been concluded that fnr gene is expressed under both aerobic and anaerobic

conditions and is subject to auto-regulation and repression by glucose, particularly during anaerobic growth (Spiro and Guest, 1987b; Pascal et al., 1986). These findings imply that during anaerobiosis the FNR protein adopts an active conformation, in which it functions both as a repressor of the fnr gene and as an activator of fnr-dependent genes.

iv. narL gene

Besides positive regulation by fnr in the anaerobic metabolism, another gene narL is also necessary for both positive and negative anaerobiosis regulations of a number of operons. Strains containing mutation in narL are unable to repress fumarate reductase and trimethylamine-N-oxide reductase, or to induce expression of nitrate reductase synthesis in the presence of nitrate (Stewart and MacGregor, 1982; Iuchi and Lin, 1987; Kalman and Gunsalus, 1988). The narL gene product is required to form an active repressor protein for controlling frdABCD expression and an activator for induction of narGHJI. The DNA sequence of narL has been determined (Gunsalus et al., 1989). The deduced amino acid sequence of narL is similar to other bacterial genes coding for DNA-binding proteins of the two-component regulatory systems (Nohno et al., 1989). It was proposed that narL gene product was a DNA binding protein which interacts with the gene product of narX (Kalman and Gunsalus, 1989; Stewart et al., 1989). narX, which lies upstream of narL, was supposed to encode a nitrate-sensor

protein. Besides having homology with DNA binding proteins, narL is also homologous to a number of kinases. narL may have a role in phosphorylation/dephosphorylation (Gunsalus et al., 1989). Their results also indicated that narL and narX are present in a defined ratio in the cell to elicit the effect of nitrate on some anaerobiosis inducible genes.

v. Other regulatory genes

Besides fnr and narL genes, other less well characterized genes involved in the genetic regulation of anaerobic metabolism included ntrA (Birkmann et al., 1987), oxrC (Jamieson and Higgins, 1986), oxrE (Tang and Barrett, 1986) and pflR (Wong and Kwan, 1988).

ntrA encodes a sigma factor which regulates genes involved in nitrogen metabolism (Hirchman et al., 1985). Birkmann et al. (1987) observed that the 5' non-coding region of fdhF (formate dehydrogenase H) showed homology with the promoters from genes involved in nitrogen fixation (Ausubel, 1984). They demonstrated that the anaerobic expression of fdhF in the presence of formate was ntrA dependent. The enzyme synthesis of hydrogenase 3 also showed a dependence on NtrA (Birkmann et al., 1987).

The oxrC mutant is defective in phosphoglucose isomerase activity (pgi). The oxrC gene is required for the anaerobic expression of formate dehydrogenase (FDH_H) of the formate hydrogenlyase (FHL) pathway, an anaerobically inducible tripeptidase (pepT) and hydrogenase isoenzymes 1 and 3

(Jamieson and Higgins, 1986; Jamieson et al., 1986). The oxrC mutation is shown to affect the level of negative supercoiling of plasmid DNA and its effects on gene expression can be explained as secondary consequences of altered DNA topology (Bhriain et al., 1989).

oxrE gene is a genetic locus closely linked but distinct from oxrA and linked to oxrA and shown to affect nitrate respiration of S. typhimurium (Tang and Barrett, 1986). pflR has been shown to affect the anaerobic expression of pfl (Wong and Kwan, 1988). The primary defect caused by pflR mutation is a deficiency in lactate production. narK , a gene located upstream of narGHJI, is induced by nitrate and its expression is dependent on fnr and narL (Stewart and Parales, 1988). It is speculated that narK product is a repressor since mutation in narK can relieve nitrate inhibition of formate-hydrogen lyase gene expression (Stewart and Berg, 1988).

vi. Proposed FNR and NARL recognition sequences

A consensus FNR binding site have been proposed (Spiro and Guest, 1987b; Eiglmeier et al., 1989; Jayaraman et al., 1989). By mutational analysis of the nucleotide sequence at the FNR-dependent nirB promoter in E. coli, Jayaraman et al. (1989) found some point mutations which reduced anaerobic induction. The positions of these point mutations was suggested to involve in FNR binding. By comparison of the nucleotide sequence of the nirB promoter with other five

FNR-regulated promoters (narG, frdA, pfl, aspA and fnr) in E. coli, a consensus sequences for FNR binding site is proposed. The site has a sequence of 5'-AAAaTTGATaT-3' where bases present in 50% or more of the cases are in capitals. Eiglmeier et al. (1989) analyzed Six FNR-dependent genes (frdA, glpA, dmsA, fnr, narG, nirB) and defined the transcriptional startpoints of these genes. They found that the promoters of the four FNR-dependent genes were arranged in a similar pattern. A 22bp dyad symmetry was found about 30 bp upstream of the transcriptional startpoints. The consensus sequence for the half site recognized by FNR (AAA-TTGAT) is only slightly different from that of CAP (AA-TGTGA). This comparison of FNR binding to that of CAP binding site is interesting because an altered FNR protein can activate the lac operon of E. coli (Spiro and Guest, 1987a).

The bases in the consensus sequence which involved in discrimination between FNR and CRP binding was analyzed by Bell et al. (1989a). They genetically engineered a DNA fragment carrying melRp in which the wild type CRP binding site was replaced with synthetic oligonucleotides containing either FNR or CRP binding sequences. When the synthetic oligonucleotide contains the 22 bp consensus for FNR binding sites, expression from melRp is dependent on FNR but not CRP. Single changes at either of two symmetrically-related positions create sites that are recognized by both FNR and CRP. Changes at both positions result in a site that is not

recognized by FNR but which binds CRP tightly. The consensus CRP site and FNR site and the engineered melRp sequences were listed below (Bell et al., 1989a):

Consensus CRP site	aAaTGTGAtct┼agaTCACAtTt
	center of symmetry
	5 18
pmelRD25 CF	AAATGTGATGT┼ACATCACATGG
	recognized by CRP only
pmelRD25 CF	AAATGTGATGT┼ACATCAAATGG
	recognized by both CRP and FNR
pmelRD25 FC	AAATTTGATGT┼ACATCACATGG
	recognized by both CRP and FNR
pmelRD25 FF	AAATTTGATGT┼ACATCAAATGG
	recognized by FNR only
Consensus FNR site	AAAtTTGATaT┼AtATCAAaTTT

The differences among the engineered sequences are at position 5 or 18 as indicated. It showed that symmetric T:A to G:C and A:T to C:G transversions at positions 5 and 18 respectively are sufficient to convert the FNR site (FF) which is recognized by FNR but not CRP, to a site (CC) that binds CRP tightly but not FNR. While FC site, which contains a T:A at position 5 and a C:G at position 18, binds both CRP and FNR. Similarly, both activators recognize the CF site

with a G:C at position 5 and A:T at position 18, implying that both activators can recognize sites containing one poorly-binding half site.

Similarly, comparing the promoter sequences of narG (induced by nitrate under anaerobic condition), frdA, fdhF and dmsA (repressed by nitrate under anaerobic condition) in E. coli, Eiglmeier et al. (1989) proposed a NarL-recognition site. The sequence of the NarL-recognition site is 5'-tAtttaTTtTCAgTGAaAAtaaata-3' where bases present in 50% or more of the cases are in capitals.

D. Future prospect

So far about 40 anaerobiosis-inducible genes were identified and about 16 genes are cloned and sequenced. However, only 6 of these sequenced gene have been analyzed for their 5'non-coding region. The transcriptional initiation sites of these 6 genes are clearly defined (Eiglmeir et al., 1989). All these genes were fnr-dependent. A putative consensus fnr-binding site has been suggested when the promoters of the 6 genes were aligned for homology. However, to get better homology, more anaerobiosis-inducible promoters should be included for comparison. Moreover, whether the FNR protein really binds to that suggested FNR-binding site have not been tested in vitro such as by gel retardation assay or DNA I footprinting. Besides fnr-dependent anaerobiosis-inducible promoters, there are other fnr-indepedent anaerobiosis-inducible promoters. Is

there any conserved region among the promoters of these *fnr*-independent genes ? So far, all the anaerobiosis-inducible genes which are sequenced and analysed are mainly that of *E. coli*. Is the consensus FNR-binding site (or OxrA-binding site) also present in that of *S. typhimurium* ? Hence, cloning and sequencing of anaerobiosis-inducible promoter from *S. typhimurium* can give more information about the molecular regulation of anaerobic metabolism.

The genetic regulation of anaerobic metabolism has aroused intensive study in different labatories. There are still many challenges and interesting questions to be solved.

Besides understanding the molecular regulation of anaerobic metabolism, there is also a practical value for using an anaerobiosis-inducible promoter for conditional expression of cloned genes. Actually, a new oxygen-regulated promoter plasmid called OXYPRO (induced by anaerobiosis) is now available from Exogene (Hughes et al., 1989). Hughes et al. (1989) showed that high expression levels of a cloned gene was obtained with the OXYPRO when the host cell was grown in a tightly capped test tube. The expression level was comparable to that using the *tac* promoter which requires an chemical inducer IPTG for maximal expression. Thus the use of an anaerobiosis-inducible promoter for regulated expression of cloned gene has an advantage over the currently used promoters which requires harmful temperature shift or expensive chemicals for induction.

CHAPTER 2

Isolation of anaerobiosis-inducible promoters

I. Introduction

Anaerobic induction is still observable when *pfl* (pyruvate formate lyase) was cloned in a high-copy-number plasmid (Pecher et al., 1982). Thus it seems that I can cloned promoters of anaerobiosis inducible genes in plasmids, and can still detect the induciblility of these promoters by anaerobiosis. Initially, I hope to clone the promoters of some known anaerobiosis genes. Some lambda clones containing DNA fragments at about the map location of *ack* (acetate kinase) and *tor* (trimethylamine-N-oxide reductase) in the chromosome of *S. typhimurium* were requested from Kukral et al. (1987), while that of *E. coli* were requested from Kohara et al. (1987). However, I only obtained that of *S. typhimurium* from Kukral. Unfortunately, after preliminary analysis of the lambda clones, the lambda clones were unlikely to contain the *ack* or *tor* genes of *S. typhimurium*. As a result, a "shot gun" approach was employed for the hunting of anaerobiosis-inducible promoters using promoter probe plasmids.

Two promoter probe plasmids, pKK232.8 (Brosius, 1984) and pFZY1 (Koop et al., 1987) were used for the isolation of anaerobiosis inducible promoters. At the beginning, pKK232.8 was used. But later, pFZY1 was chosen because pFZY1 have

some advantageous propertes — it is a low-copy-number plasmid and utilizes *lacZ* gene as the "reporter" gene. The detail properties of pKK232.8 and pFZY1 will be discussed in the following paragraphs.

A. Properties of promoter probe plasmid pKK232.8

Plasmid pKK232.8 (Figure 1) is a derivative of pBR322 (Bolivar et al., 1977). The plasmid has a promoterless CAT gene (chloramphenicol resistance). Insertion of a promoter in one of the polylinker sites confers chloramphenicol resistance to the host. An additional feature in pKK232.8 is the insertion of a DNA fragment that places translational stop codons in all three reading frames between the polylinker and the ATG start codon of the CAT gene. As a result, it is a transcriptional fusion vector. The *rrnB* transcription terminator T1 in front of the polylinker sites prevent plasmid-originated clockwise transcription into the CAT structural gene and since the terminator functions to some extent in the opposite orientation it will also reduce counterclockwise transcription from an inserted divergent promoter into the ß-lactamase structural gene. Two terminator fragments (T1 and T2 from *rrn*B) are also inserted in the downstream region of the CAT gene. The use of pKK232.8 shows an excellent correlation of promoter activity and levels of chloramphenicol resistance or CAT activity.

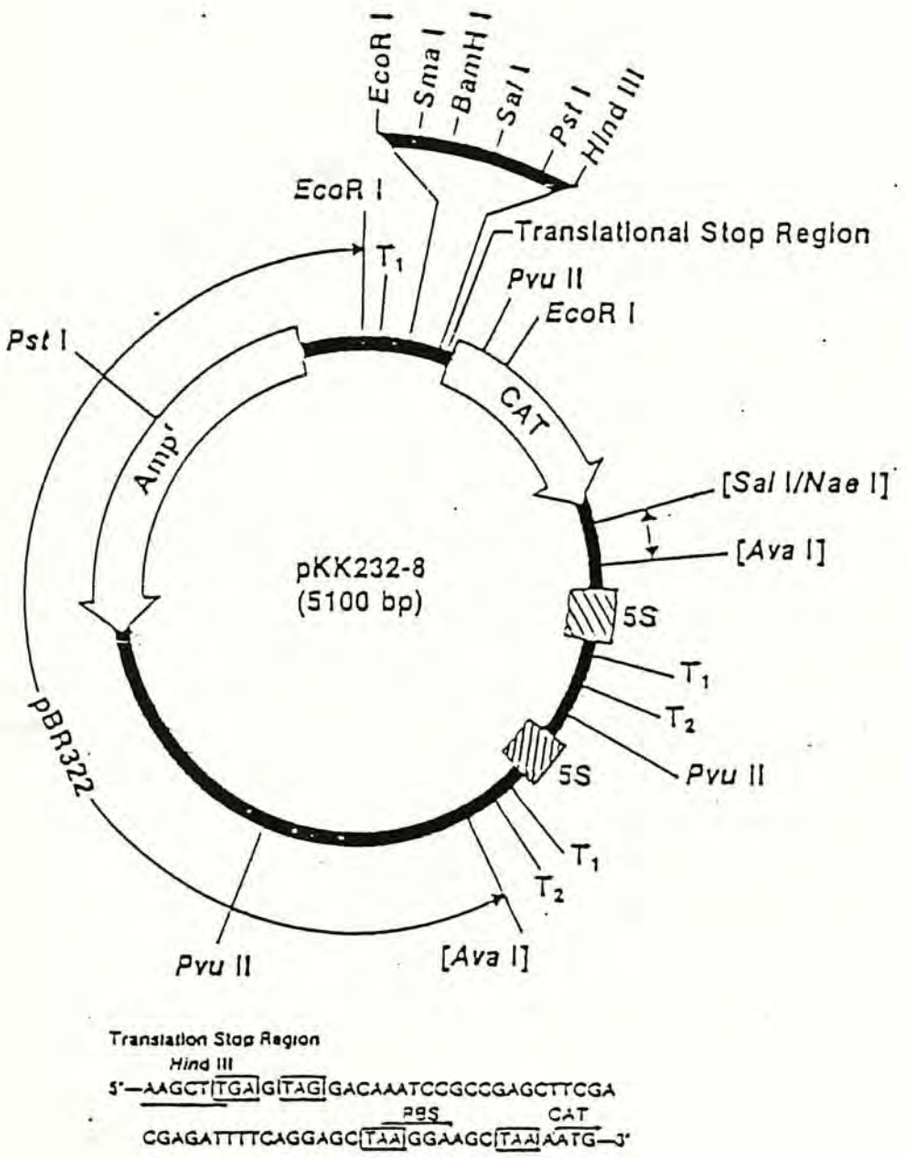


Figure 1. Restriction Map of promoter probe plasmid pKK232.8 (Brosius, 1984)

B. Properties of promoter probe plasmid pFZY1

Plasmid pFZY1 (Figure 2) is a low-copy-number vector containing the oriF replication origin and without the par locus. It presents in the cell in one to two copies per genome (Koop et al., 1987). The plasmid contains the multiple restriction site linker of M13mp18 upstream from a promoterless ß-galactosidase-coding lacZ gene. Similar to pKK232.8, plasmid pFZY1 also contains stop codon in all three reading frames and necessary translational signal in front of the lacZ structural gene. It can be retained in the host by the presence of ampicillin, and each inserted promoter gives rise to ß-galactosidase activity. Koop et al. (1987) showed that the regulation of the wild-type lac promoter and mutants in pFZY1 was similar to that observed for lac promoters in the chromosome.

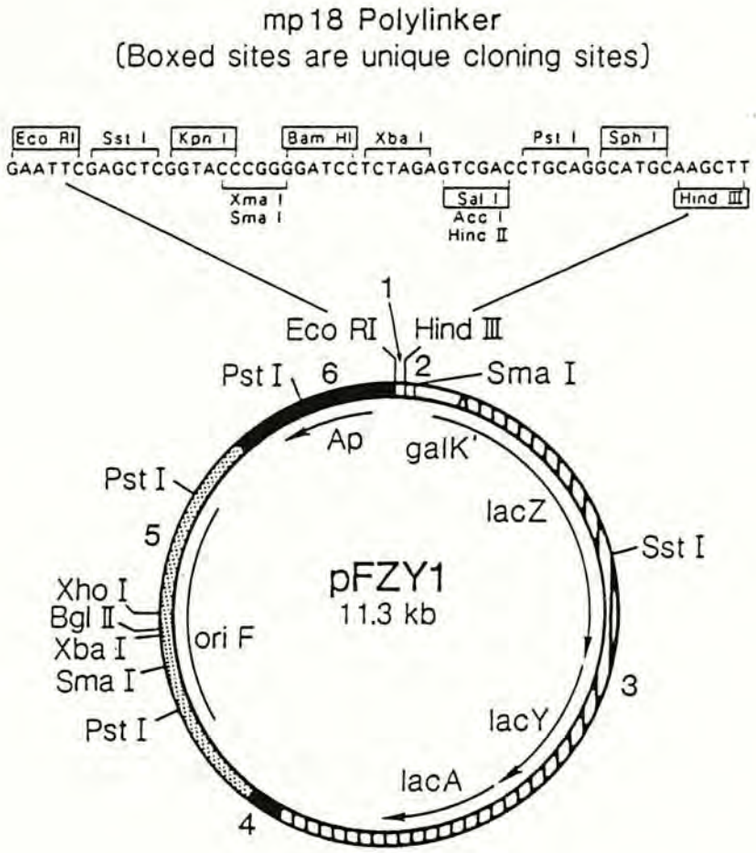


Figure 2. Restriction Map of promoter probe plasmid pFZY1 (Koop et al., 1987)

II. Materials and methods

A. Bacterial strains and plasmid. Chromosomal DNA was prepared from *S. typhimurium* LT2 and *E. coli* K12. Competent cells were prepared from *E. coli* strains JM107 (Yanisch-Perron et al., 1985) or MC1061-5 (Koop et al., 1987). Promoter probe plasmid pKK232.8 (Brosius, 1984) was purchased from Pharmacia-LKB Biotechnology, and pFZY1 (Koop et al., 1987) was a gift from Dr. Bourgeois of Salk Institute, La Jolla, U.S.A.

B. Media. Cells were grown in Luria broth (LB, 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl per liter broth) with ampicillin (50 μg/ml) for cell with plasmid pKK232.8 or pFZY1. For plate, 15 g/l Bacto-agar was added. Chloramphenicol was added at various concentrations when appropriate. LG medium was LB with 0.2% D-glucose. LGE medium was LG medium buffered with E medium. For preparation of competent cells, SOB medium was used. SOB medium consisted of 2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 10mM $MgSO_4$. SOC medium is identical to SOB medium but contains 20mM glucose in addition. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were from Difco Laboratories. Antibiotics were purchased from Sigma Chemical Company.

C. Solutions.

1.SET buffer: 150 g .sucrose was dissolved in double distilled water. 50 ml 1M Tris-HCl (pH 8) and 100 ml 0.5 M EDTA were added. The final volume was brought to 1 liter by adding distilled water.

2.Alkaline lysis buffer: 0.2 N NaOH, 1% SDS was freshly prepared by mixing 1 ml 2N NaOH and 0.5ml 20% SDS, and the volume was brought to 10 ml.

3. 3M Sodium acetate, pH 4.8. 408 g sodium acetate.$3H_2O$ was dissolved in 800 ml H_2O and the pH was adjusted to pH 4.8 with glacial acetic acid and added water to 1 liter.

4. TE buffer: 10 mM Tris-HCl, 1 mM EDTA in water, prepared from 1 M Tris buffer pH 8.0 and 0.5 M EDTA stock solutions..

5. Phenol: solid phenol was redistillated and saturated with TE buffer (pH 8). The phenol was stored in freezer and melted before used for phenol extraction of nucleic acids.

6. Gel-loading buffer. The buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol and 30% glycerol in water was prepared.

7. 0.5 M EDTA (pH 8.0). 186.1 g $Na_2EDTA.2H_2O$ was dissolved in 700 ml H_2O and pH was adjusted to 8.0 with 10 N NaOH (about 50 ml) and added water to 1 liter.

8. 1 M Tris.Cl (pH 8.0). 121 g Tris base was dissolved in 800 ml H_2O and pH was adjusted to 8.0 with concentrated HCl

and added water to 1 liter.

9. TBE electrophoresis buffer. 5X stock solution was prepared and diluted for use. The 5x stock contained 54 g Tris base, 27.5 g boric acid and 2 ml 0.5 M EDTA in 1 liter distill water.

10. TAE electrophoresis buffer. 10X stock solution was prepared and diluted for use. The 10X stock contained 48.4 g Tris base, 11.4 ml glacial acetic acid and 7.44 g $Na_2EDTA.2H_2O$ in 1 liter distill water.

11. 2X KGB buffer (Hanish and McClelland, 1988) consisted of 200 mM Potassium glutamate, 50 mM Tris-acetate(pH7.6), 20 mM magnesium acetate, 100 µg/ml bovine serum albumin, 1 mM 2-mercaptoethanol (filter-sterilized and stored at 4°C).

D. Small scale preparation of plasmid DNA (Birnboim and Doly, 1979). Single colony was picked and grown up as 2 ml overnight culture in LB. One and a half ml of culture was transferred to an Eppendorf tube and pelleted with a microcentrifuge for five minutes. Cell pellet was resuspended in 1 ml SET buffer. The resuspended cell pellet was washed with SET buffer, pelleted again and resuspended in 0.15 ml SET. Five µl RNase A (10 mg/ml, boiled for 2 minutes to inactive any DNase activity) was added and left in ice for 10 minutes. Alkaline lysis buffer (350µl) was added and mixed gently by inverting the tube several times until lysis of cell observed. The clear lysate was placed on ice for 5

minutes, and then 250 µl 3M Sodium acetate (pH 4.8) was added. The mixture was mixed gently by inverting the tube several times until white precipitate (SDS-protein-chromosome complex) was observed. The tube was left on ice for 10 minutes. The precipitate was pelleted by centrifuging for 10 minutes in a microcentrifuge. Supernatant was transferred to a new tube and 750 µl ice cold anhydrous isopropanol was added to precipitate DNA. The tube was left at room temperature for 5 minutes, and then the DNA was pelleted by centrifuging 10 minutes with maximum speed. The DNA pellet was washed once with 80% ethanol briefly and vacuum dried. The plasmid was resuspended in 10 µl double distillated water. For pKK232.8, about 1 µg DNA was obtained, but for pFZY1, only about 0.2 µg DNA was obtained.

E. Large scale preparation of plasmid DNA.

The alkaline lysis procedure as described by Birnboim (1983) was used.

1. Growth of culture

Cell harboring the required plasmids were checked first for the presence and sizes of the plasmids with small scale prepared plasmid DNA before large scale preparation. Five ml LB-Amp^{50} medium was inoculated with a single colony of E. coli containing the desired plasmid. The culture was grown overnight with vigorous shaking at 37°C. Then 1 ml of the 5ml overnight culture was transferred to 500 ml LB

medium in a 2 liter flask and grown overnight at 37°C with vigorous shaking. For low-copy-number plasmid, pFZY1, 200 µg/ml of ampicillin was added to the LB medium .

2. Alkaline lysis with NaOH-SDS solution

The cultures were centrifuged in 500ml bottle with Sorval GS3 rotor and resuspended in 20 ml SET buffer. Then 40 ml NaOH-SDS solution was added, mixed and left at room temperature for 5 min for cell lysis. To the viscous lysate, 30 ml 3M potassium acetate solution was added, mixed gently and left at room temperature for 10 min. The flocculent precipitate was then removed by centrifugation at 7000 rpm in 500ml bottle or 12000rpm in 250ml bottle with GSA rotor. Supernatant was transferred to a new bottle and equal volume of isopropanol was added. Precipitated DNA was recovered by centrifugation at 1200rpm with Sorval GSA rotor for 20 min. The DNA pellet was dried and dissolved in 4 ml TE buffer.

3. CsCl/ethidium bromide banding and purification of plasmid.

CsCl (4.4g) was added to the 4 ml DNA solution. After the CsCl was dissolved, 0.4 ml 10mg/ml ethidium bromide solution was added. The density of the solution was measured by weighing 1 ml of the solution and adjusted to a density between 1.55 and 1.59 g/ml. The CsCl/EtBr DNA solution was added to 5ml polyallomer tubes, overlaid with mineral oil when using Beckman SW50.1 rotor or to 10 ml polyallomer tube with Beckman Type 50 rotor. The plasmid was

banded by centrifugation for 48 hours at 42,000 rpm at 20°C. After centrifugation, the plasmid band was recovered carefully by suction with a 1 ml syringe and a 19-gauge needle. Ethidium bromide was removed by extraction with water-saturated n-butanol several times. The plasmid DNA was precipitated by adding 2 volume of TE buffer and 6 volume of 100% ethanol. Precipitated plasmid was removed by centrifugation at 10,000 rpm for 10 min, washed with 80% ethanol, dried and redissolved in 0.5 ml 0.3M Sodium acetate solution. To the resuspended plasmid solution in 0.3 M sodium acetate in 1.5 ml Eppendorf tube, 1 ml 100% ethanol was added to reprecipitate the plasmid again so as to remove trace amount of CsCl. Precipitate plasmid DNA was pelleted by spinning in microcentrifuge for 10 min at 12,000rpm. The pellet was rinsed with 80% ethanol, dried, dissolved in TE and measured concentration of DNA by determining absorbance at 260 nm. (1 OD_{260} = 50µg/ml).

F. Digestion of DNA with restriction endonucleases. In a typical reaction, 1 µg of DNA was digested in 20µl appropriate digestion buffer with 1 unit or more of restriction enzyme at the appropriate temperature for 1 hour or longer in a sterilized Eppendorf tube. The reaction was stopped by heating at 65°C for 30 minutes for heat sensitive restriction enzymes or by addition of 1/10 volume of 0.2M EDTA (pH 8.0). If the restricted DNA was to be purified, it was extracted once with phenol, chloroform and

isoamylalcohol (25:24:1) once and precipitated with ethanol. Restriction enzymes used were purchased from New England Biolabs Inc., Amersham Corp., or Promega Biotec.

G. Analysis of DNA samples with agarose gel electrophoresis. DNA samples were separated in agarose gel with agarose concentration appropriate for the size of DNA fragments to be separated. Generally, a 0.7% agarose gel was prepared for the analysis of 0.8 to 12 kb DNA fragments, 1.2 % for the analysis of 0.4 to 7 kb DNA fragments, and 0.5% for the analysis of 1 to 30 kb DNA fragments. The buffer used for gel preparation and electrophoresis buffer was 1X TBE buffer. If the separated DNA fragments was to be recovered, 1X TAE buffer was used instead of TBE.

To 10µl DNA sample, 2 µl loading buffer was added. The sample was loaded into the well of the agarose gel and the voltage was run at 1 to 10 V/cm of gel. The process of separation was monitored by the migration of the dyes in the loading buffer. After the running, the DNA was visualized by staining the agarose gel in ethidium bromide solution (0.5µg/ml) for 30 min and destained in water for an additional 30 min. Alternatively, 1µl ethidium bromide solution (10mg/ml) was added to the melted agarose solution during the agarose gel casting. The ethidium bromide stained DNA fluoresced in the agarose gel when illuminated by UV transilluminator and was photographed with Polaroid MP4 camera with Polariod type 667 film (ASA 3000). Restriction enzyme HindIII digested lambda DNA was used as a DNA size markers.

Agarose was purchased from FMC Marine Coloids.

H. Dephosphorylation of DNA fragments. Dephosphorylation of DNA fragments was carried out in 20 µl CIP (calf interstinal alkaline phosphatase) buffer with 1-5 µg linear DNA and 2 units of calf intestinal alkaline phosphatase (Promega). To dephosphorylate protruding 5' termini, the reaction mixture was incubated at 37°C for 30 minutes. Another 2 units of CIP was added and the reaction mixture was incubated for a further 30 minutes.

To dephosphorylate DNA fragments with blunt ends or recessed 5' termini, the reaction mixture was incubated for 15 minutes at 37°C and 15 minutes at 56°C. Then another 2 units of CIP was added and the incubation repeated at both temperature.

After incubation, the reaction mixture was extracted with phenol, chloroform and isoamyl alcohol (25:24:1). The aqueous fraction were added 1/10 volume of 3 M sodium acetate, 2 volume of 100% ethanol and mixed well. The mixture was kept in -20°C overnight or -70°C for one hour and centrifuged in an Eppendorf microcentrifuge for 15 minutes at 4°C. The DNA pellet was washed with cold absolute ethanol, dried under vacuum, and then resuspended in TE buffer.

I. Partial digestion of chromosomal DNA with restriction enzyme Sau3A. Partial digestion of chromosomal DNA was used to generate DNA fragments of different sizes. The reaction

was carried out in 50 µl containing 50 mM NaCl, 10 mM Tris.Cl (pH7.5), 10mM $MgCl_2$, 1mM dithiothreitol, 10 µg chromosomal DNA and different amount of restriction enzyme Sau3A (1 Units, 0.8 units, 0.4 units and 0.2 units). The reaction mixtures were incubated for 1 hour at 37°C and reaction was stopped by incubated the mixture at 65°C for 30 minutes. Digested DNA was analyzed by agarose gel electrophoresis. Digested DNA sample with desired size was chosen for ligation with BamH1 digested promoter-probe plasmids.

J. Ligation of DNA. DNA fragments were ligated to the vector DNA in a molar ratio of 1:1 in 20 µl ligation buffer (or 0.5X KGB buffer) with 1 mM ATP and 1 unit of T4 ligase (Amersham). Reaction mixtures were incubated at 16°C, 15 hours for cohesive ends, and 20 hours or longer for blunt ends (or incubated at room temperature for 15 hours with 0.5 mM ATP in the reaction mix). After ligation, the ligated DNA in the reaction mixture was directly used for transformation without any purification.

K. Preparation of competent cells.

1. Competent cells for heat shock transformation

Competent cells were prepared according to the Method of Hanahan (1985). A colony from a freshly streaked SOB agar plate was picked and grown in 10 ml SOB medium. When the cell density reached 10^8 cells/ml, 1 ml of the culture was

transferred to 100ml SOB broth in 1 liter flask. The culture was incubated at 37°C with moderate agitation until the cell density reached 4-7x10^7 cells/ml. The cells were transferred to 250ml centrifuge bottle and chilled on ice for 15 minutes before centrifugation. The cell was then pelleted by centrifugation at 3000rpm with GSA rotor. The pellet was resuspended in 30ml RF1 buffer and incubated on ice for 30 minutes. Cells were pelleted again and resuspended in 8 ml RF2 buffer and incubated on ice for 15 minutes. Aliquots of cell suspension of 200 µl were distributed into 1.5 ml Eppendorf centrifuge tubes which were placed at -70°C for later use.

2. Electro-competent cells for high efficiency electro-transformation (Dower et al., 1988).

Half liter of L-broth in 2 liter flask was inoculated with 0.5 ml fresh overnight culture and grown at 37°C with vigorous shaking to an absorbance at 600nm of 0.5. To harvest the cells, the flask was chilled on ice for 15 minutes, and then centrifuged in a cold Sorval GS3 rotor at 3500rpm for 10 minutes. Cell pellets were resuspended in 500ml of a cold, low ionic strength wash medium (1 mM Hepes, pH 7). The suspension was centrifuged again and cell pellets resuspended in 250ml wash medium (1mM Hepes, pH 7). After the wash, the cells were centrifuged and resuspended in 10ml 10% glycerol. The 10 ml cell suspension in 10% glycerol was centrifuged again and resuspended in 500µl 10% glycerol. Aliquots of 40 µl of the 500µl cell suspension in 10%

glycerol was distributed into 1.5ml Eppendorf tubes, frozen on dry ice, and stored in -70°C.

L. Transformation.

1. Heat shock transformation of competent cells prepared by the method of Hanahan (1985).

Competent cells in Eppendorf tubes from -70°C freezer were allowed to thaw at room temperature until the cells suspension was just liquidified. To every 200µl competent cells, DNA solution of volume of less than 20µl was added. The tube was placed on ice for 10-60 minutes, and then transferred to water bath at 42°C for 2 minutes. After the heat shock, the tube was placed back on ice and 800µl SOC medium was added and incubated at 37°C for 30 minutes with moderate agitation. The transformed cells were plated directly or diluted before plating on selection plates.

2. Electoporation.

Electro-competent cells were thawed at room temperature. One to two µl DNA (in TE buffer) was added to each 1.5ml Eppendorf tube containing 40µl electro-competent cells, mixed well and let sit on ice for 1 minute. The Gene Pulser apparatus (Bio-Rad) was set at 25µF and 2.5 KV and the Pulse controller to 200Ω. The mixture of cells and DNA was transferred to the bottom of a cold, 0.2 cm electroporation curvette. A pulse at the above settings was given. One ml of SOC medium was immediately added to the curvette and the content was transferred to a test tube for

moderately shaking at 37°C for 30 minutes. Cells were diluted and plated on selective medium.

M. Chloramphenicol resistance test for promoter clones in plasmid pKK232.8. The level of resistance to chloramphenicol was determined semiquantitatively by streaking transformants on LB plus 0.2% glucose agar plates containing increasing concentrations of chloramphenicol. Two set of plates were used: one set was incubated aerobically, and the other set was incubated anaerobically at 37°C. Growth was compared with that on a control plate without chloramphenicol and scored as resistant (similar colony size), or sensitive (smaller colony size or no growth at all). Anaerobic promoter was identified as transformants having higher resistance anaerobically.

N. Preparation of crude cell extract for chloramphenicol acetyltransferase (CAT) assays. One hundred µl fully grown overnight culture in LB plus ampicillin (50 µg/ml) was inoculated into 10 ml LB plus 0.2% glucose and ampicillin (50 µg/ml) in duplicate. One set of cultures were grown aerobically in a rolling drum at 37°C and the other set was grown in anaerobic jars (BBL Microbiology systems), using an atmosphere of 95% H_2 - 5% CO_2 at 37°C. Cells were allowed to grow for 6 to 10 hours. Then the cells were spinned down at 4°C and resuspended in 1 ml 100mM Tris buffer (pH7.8), which was left at -20°C overnight. The resuspended cells were sonicated with a ultra-sonic disintegrator (Sonicator

Cell Disrupter Model W 200R, Heat System-Ultrasonic, Inc, Plainview, N.Y.) fitted with a microtip (2mm) probe. During sonication, cell suspension was placed on ice and sonicated at full power for 30 seconds twice with 1 minute cooling period interval. Cell debris was removed by centrifugation and the crude cell extract was immediately assayed for CAT activity and protein content.

O. CAT assay. The assay for CAT was a modification of the spectrophotometric method of Shaw (1975). The reaction mixture was freshly prepared by dissolving 8 mg of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Sigma) in 2.0 ml 1.0 M Tris-HCl buffer (pH 7.8), adding 0.4 ml acetyl-CoA from a 5 mM frozen stock solution, and the total volume was adjusted to 20ml with distilled water. To 880 µl reaction mixture, 100µl sample was added. The A_{412} was adjusted to zero and then measured for 30 second for background CAT activity. To the enzyme containing reaction mixture, 20µl of 5 mM chloramphenicol (dissolving in 70% ethanol, final concentration 0.1 mM) was added to start the reaction. The increase in absorbance (A_{420}) was recorded for about 5 minutes. The CAT enzyme specific activity (in nmol/min/mg protein) was calculated as increase A_{420}/min/0.0136/mg protein.

P. Protein assay. Protein was determined by the method of Bradford (1976) with the Bio-Rad protein assay reagents. Several dilutions of protein standard containing from 0.2 to

1.4 mg/ml was prepared for a standard curve. To 0.1 ml of standards, appropriately diluted samples, and sample buffer in clean, dry test tubes, 5.0 ml diluted dye reagent was added to each test tube and mixed. After a period of from 5 minutes to one hour, A_{595} of each test tube was measured with a 1 cm curvette versus reagent blank. A_{595} of the standards was plotted against their concentration. Protein concentration of the unknown was read from the standard curve.

Q. ß-galactosidase assay. The assay procedure was similar to that described by Miller (1972). Each strains (promoter clone with plasmid pFZY1) to be assayed for ß-galactosidase activity was first grown overnight in LB plus ampicillin (50µg/ml) at 37°C in rolling drum. Fully grown cultures were, inoculated into culture tubes of test media t an inoculation level of 1%. aerobic cultures were grown in 1 ml test medium in 16mmx100mm test tube shaking rigorously in rolling drum. Anaerobic culture was grown in anaerobic jar. The cultures were grown for 6 hours. After growth, absorbance of the cultures were measured at 650nm against a medium blank. The cultures were kept in ice and immediately prepared for assay.

For enzyme assay, 7.5µl toluene was added to an 0.5 ml culture. The culture was vortexed vigorously for 15 seconds. Then the culture was left at 37°C for 30 minutes to evaporate toluene and then put back on ice. To 1.1 ml Z assay buffer in a clean test tube, 0.1 ml toluened sample

was added and equilibrated at 30°C in a thermal block. 0.3 ml ONPG (Sigma, o-nitrophenyl galactoside, 4mg/ml in 0.1 M phosphate buffer, equilibrated at 30°C) was added to the mixture to start reaction an zero time was recorded at this instant. When the yellow color formed in the reaction mixture was sufficient to produced an absorbance at 420nm of 0.3 to 0.9, the reaction was stopped by adding 0.75ml 1 M Na_2CO_3 to the reaction mixture. The tube was then left on ice for more than 10 minutes before the absorbance at 420 nm was measured with a 1 cm curvette.

Unit activity of the ß-galactosidase was expressed as follows:

A_{420}/ml lysed cell = A_{420} x 2.25/0.1

Where 2.25 was the finally assay mixture volume with Na_2CO_3 added, and 0.1 was the volume of sample for assay.

Since 1 nmole/ml o-nitrophenol(ONP) corresponds to 0.0045 absorbance unit at 420 nm, A_{420}/ml lysed cells was thus expressed as:

nmoles of ONP/ml = A_{420}/ml x 1/0.0045 nmoles

Rate of ONP formation was thus;

$$\text{nmoles of ONP/ml.min} = \frac{\text{nmole of ONP/ml}}{\text{time of incubation (min)}}$$

specific activity was expressed as: $\frac{\text{nmoles of ONP/ml.min}}{A_{650}}$

III. Results

A. Molecular cloning of anaerobiosis-inducible promoters with promoter-probe plasmid pKK232.8

Promoter probe plasmid pKK232.8 was initially used for the cloning of anaerobiosis-inducible promoters from S. typhimurium. Several trials were attempted and different batches of Sau3A-partial-digested DNA fragments were used for the construction of recombinant plasmid with pKK232.8

The result of Sau3A partial digestion of S. typhimurium chromosomal DNA was shown in Figure 3. Various sizes of DNA fragments were generated. For the first trial, the batch of Sau3A digested DNA with major DNA fragment sizes about 6 kb was used for ligation with BamH1 digested plasmid pKK232.8. The recombinant plasmids were used to transform competent cell JM107. Transformants were directly selected on LB-Amp^{50}-Cm^{50} agar plate incubated anaerobically for anaerobic expressed CAT activity from transformants. The transformation efficiency was exceptionally low. Only few anaerobically expressed promoter clones were recovered. The selection procedure was thus changed. Recombinant plasmids were transformed into E. coli JM107. Transformants were selected first on LG-Amp^{50} medium aerobically. Then the transformants were replica-plated onto LG-Cm^{50} plate and allowed to grow anaerobically. Glucose added at 0.2% to LB

Figure 3. Sau3A partial digestion of Salmonella typhimurium chromosomal DNA. Lane A was HindIII digested λ DNA as molecular size markers. Amount of Sau3A used for digestion in each lanes: B, 1µg DNA/0.1 U; C, 1 µg DNA/0.08 U; D, 1 µg DNA/0.06 U; E, 1 µg DNA/0.04 U; F, 1 µg DNA/0.02 U; G, no enzyme added.

medium could increase the anaerobic growth. Moreover, the medium was buffered with E medium. A total of 126 anaerobic promoter clones were recovered as colonies grown anaerobically on LGE-Cm^{50} plate. These 126 colonies were tested for their strength by streaking them on different concentration of chloramphenicol. The result was given in Table 2.

Table 2. Preliminary study of chloramphenicol sensitivity of 126 promoter clones under anaerobic condition[a]

Cm[b] (μg/ml)	no. of clones resistant to that concentration
300	35
200	48
150	63
100	77
50	126

a. LGE-Amp^{50} was used as the basal medium .

b. Concentration of chloramphenicol (Cm) in LGE-Amp^{50} plate.

After the preliminary study of the 126 clones for anaerobic chloramphenicol sensitivity, the concentration of chloramphenicol at 150µg/ml was arbitrary chosen for the test of difference in aerobic and anaerobic sensitivity. One of the clones, Strain HSK1501 (with recombinant plasmid pHSK1), differed in its resistance to chloramphenicol at 150µg/ml under anaerobic and aerobic conditions. The clone was resistant to 150 µg/ml chloramphenicol when grew anaerobically but sensitive when grew aerobically. This was a putative anaerobiosis-inducible promoter clone.

After the preliminary study, three independent transformations with recombinant pKK232.8 plasmid were performed, but the batch of Sau3A digested DNA used was of smaller DNA fragments size (about 2 kb). Moreover, the competent cell E. coli strain MC1061-5 was used instead of JM107. It was because the strain MC1061-5 in our hand had a greater transformation efficiency than JM107. Transformation efficiency is generally about 10^6/µg DNA for MC1061-5. The number of anaerobic chloramphenicol resistant transformants isolated and the putative anaerobiosis-inducible promoter containing recombinant plasmids obtained in each trial were shown in Table 3.

Table 3. Number of anaerobic expressed promoter clones isolated and putative anaerobiosis inducible promoters obtained[a].

Trial	no. of anaerobic resistant transformants	putative anerobiosis-inducible promoter containing plasmid
1st	600	pHSK2
2nd	182	pHSK4, pHSK7
3rd	350	pHSK8, pHSK9

a. anaerobic resistant transformants were picked and patched onto two LGE-Amp^{50}-Cm^{150} plates. Poor aerobic growth but good anaerobic growth was regarded as putative anaerobiosis-inducible promoter-clones.

Table 3 showed the results of three independent transformation using competent cell MC1061-5. Transformants were first selected for ampicillin resistance aerobically on LGE-Amp^{50} plates and replicated onto LGE-Amp^{50}-Cm^{150} plates and grew anaerobically. From the first trial, totally 600 colonies appeared on the plates after anaerobic incubation for 24 hours. All the colonies were then picked and streaked onto two same kinds of agar plates, one incubated aerobically and the other anaerobically. One promoter clone with recombinant plasmid pHSK2 showed obvious difference in growth under aerobic and anaerobic condition, and regarded as putative anaerobiosis-inducible promoter clone. Similarly, anaerobiosis-inducible promoter containing recombinant plasmids pHSK4 and pHSK7 were identified in the second trial and pHSK8 and pHSK9 identified in the third trial. All putative anaerobiosis-inducible promoter clones were tested for the presence of recombinant plasmids, size of insert, and chloramphenicol acetyl transferase activities under aerobic and anaerobic conditions. Mini-preparation of plasmids from the clones were digested with restriction enzymes (Figure 4) and the total sizes of the digested fragments were estimated with the HindIII digested lambda DNA markers. The insert size was calculated by subtracting the size of plasmid pKK232.8 (5.2 kb). The result was shown in Table 4.

Figure 4. Gel electrophoresis of restriction enzymes digested plasmid pHSK1, pHSK2, pHSK4, pHSK7, pHSK8, pHSK9. Lanes A, H, M and R were HindIII digested λ DNA as molecular size markers. pHSK9: lane B, uncut; lane C, pstI digested. pHSK8: lane D, EcoR1 digested; lane E, SmaI digested; lane F, EcoR1 and HindIII digested; lane G, HindIII digested. pHSK7: lane I, uncut; lane J, SalI digested. pHSK4: lane K, uncut; lane L, SalI digested. pHSK2: lane N, uncut; lane O, SalI digested. pHSK1: lane P, PvuII digested; lane Q, SalI digested.

Table 4. Size of DNA insert cloned into pKK232.8 vector, and CAT activities under aerobic and anaerobic conditions.

Plasmid[a]	size of DNA insert (kb)	CAT sp act (U/mg protein)[b]		Induction ratio ($-O_2/+O_2$)
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	
pHSK1	7.1	0.021	0.650	31
pHSK2	1.4	0.380	0.710	1.9
pHSK4	4.4	0.026	0.067	2.6
pHSK7	3.9	0.014	0.080	5.7
pHSK8	3.6	0.051	0.550	11
pHSK9	4.3	0.006	0.065	11
pKK232.8	0	0	0	

a. plasmid was transformed in E. coli MC1061-5 for assay.

b. One unit of chloramphenicol acetyl transferase (CAT) is defined as 1 μM of chloramphenicol acetylated per min at 37°C. Cultures were grown in LGE-Amp[50] medium.

B. Molecular cloning of anaerobiosis-inducible promoters with promoter-probe plasmid pFZY1

Since the promoter probe plasmid pKK232.8 is a high-copy-number plasmid (about 40 copy per cell), the promoter activities may not reflect real anaerobiosis-inducible activities. Hence a low-copy-number plasmid (pFZY1) was used when it was obtained from Dr. Bourgeosis. The low-copy-number promoter probe plasmid utilizes promoterless lacZ gene as a marker for promoter analysis.

1. Use of plasmid pFZY1 for cloning E. coli anaerobiosis-inducible promoters.

Sau3A-partial-digested Escherichia coli chromosomal DNA was ligated to BamH1 digested plasmid pFZY1. Batch of Sau3A-partial-digested fragment has a major size of about 2 kb. The recombinant plasmids were used to transform competent cells of MC1061-5. Transformed cells were selected for ampicillin resistance in LB plate with X-gal and incubated aerobically at 37°C for 24 hours. Transformants appeared as blue colonies were assumed to be promoter clones. About 30 colonies were picked and purified in the same kind of agar plate. The ß-galactosidase activity of the transformants were assayed after aerobic and anaerobic growth. However, these clones did not have any obvious anaerobiosis-induced ß-galactosidase activities. Then transformation were performed again. Transformed cells were plated onto LB-Amp^{50}-X-gal. The plates were incubated

in anaerobic jars for 30 hours at 37°C. The plates were taken out and placed in refrigerator at 4°C for 24 hours. Some colonies appeared blue after incubation in refrigerator for 24 hours. Totally 92 blue colonies on the X-gal plate were picked and purified. Their ß-galactosidase activities were assayed after aerobic and anaerobic growth (Table 5). The aerobic and anaerobic activities were analyzed and presented separately as Table 6 and Table 7. The ß-galactosidase activities varied from several units to over thousands. Nine clones had higher anaerobic ß-galactosidase activity and the highest was about 10-fold increase (ratio of anaerobic activity to aerobic activity). On the hand, among these 92 clones, 13 clones had higher aerobic ß-galactosidase activities with (aerobic activity/anaerobic activity) greater than 5-fold, 33 clones with aerobic ß-galactosidase activty of 2- to 5-fold increase. The rest of the clones (37) was regarded as constitutive with similar aerobic and anaerobic ß-galactosidase activities. Only two putative anaerobiosis-inducible promoter clones (10-fold anaerobic induction) with recombinant plasmid pFE29 and pFE117 were selected for further study. The aerobic and anaerobic activity of the these two cloned promoter were given in Table 8. The plasmids pFE29 and pFE117 were digested with restriction enzyme and analysed by agarose gel electrophoresis (Figure 5). The size of the DNA insert determined for pFE29 and pFE117 were 1.6 kb and 2.9 kb respectively.

pFE29: Lanes A, B, C, D, E, F, H, I

pFE117: Lanes J, K, L, N, O, P, Q, R

HindIII-digested Lambda standards: G, M

Figure 5. Gel electrophoresis of restriction enzyme digested pFE29 and pFE117. Restriction enzyme digested pFE29 and pFE117 were analysed in 0.7% agarose gel. The direction of electrophoresis was from top to bottom. The sizes of standards were indicated in base pairs. Resstriction enzymes used in different Lane: A and J, BamH1; B and K, EcoR1; C and L, SalI; D and N, KpnI; E and O, SalI; F and R, SmaI; H and Q, SphI; I and R, uncut.

Table 5. ß-galactosidase specific activity for Escherichia coli strain MC1061 carrying recombinant pFZY1 plasmids containing promoters from Escherichia coli K12.

Plasmid	ß-Galactosidase sp act[b]		Fold induction	
	Aerobic($+O_2$)[a]	Anaerobic($-O_2$)	$-O_2/+O_2$	$+O_2/-O_2$
pFZY1	18	13	0.7	1.4
pFE1	370	31	0.08	11.9
pFE25	540	67	0.1	8.0
pFE27	200	38	0.2	5.2
pFE29	160	1600	10.0	0.1
pFE30	110	57	0.5	1.9
pFE40	100	46	0.5	2.2
pFE43	140	87	0.6	1.6
pFE44	240	190	0.8	1.2
pFE46	110	58	0.5	1.9
pFE59	17	17	1.0	1.0
pFE60	16	50	3.0	0.3
pFE61	16	17	1.1	0.9
pFE62	16	17	1.1	0.9
pFE63	21	15	0.7	1.4
pFE65	17	17	1.0	1.0
pFE68	120	20	0.2	6.0
pFE77	43	13	0.3	3.3
pFE78	460	190	0.4	2.5
pFE79	17	18	1.0	1.0
pFE80	120	130	1.2	0.9
pFE85	230	120	0.5	1.8
pFE88	210	27	0.1	7.5
pFE89	18	16	1.0	1.0
pFE90	17	16	1.0	1.0
pFE91	17	15	0.9	1.1
pFE92	16	15	0.9	1.1
pFE105	58	60	1.0	1.0
pFE110	210	200	1.0	1.0
pFE117	120	1200	10.0	0.1
pFE118	41	35	0.8	1.2
pFE126	370	150	0.4	2.5
pFE131	160	66	0.4	2.5
pFE136	23	61	2.6	0.4
pFE148	120	52	0.4	2.4
pFE149	110	27	0.2	4.2
pFE161	170	55	0.3	3.0
pFE214	32	9	0.3	3.7
pFE227	100	63	0.6	1.6
pFE229	60	39	0.6	1.6
pFE230	100	13	0.1	7.5
pFE231	110	60	0.6	1.8
pFE242	230	91	0.4	2.5
pFE244	53	17	0.3	3.2
pFE254	28	13	0.5	2.2
pFE257	61	29	0.5	2.1
pFE258	28	10	0.4	2.8

Table 5 cont'd

pFE259	66	17	0.3	3.8
pFE280	46	28	0.6	1.6
pFE288	41	17	0.4	2.5
pFE289	300	89	0.3	3.4
pFE290	1700	130	0.08	12.5
pFE291	56	120	2.1	0.5
pFE293	1200	250	0.2	5.0
pFE295	180	130	0.7	1.4
pFE296	140	15	0.1	8.8
pFE297	30	87	2.9	0.3
pFE304	200	56	0.3	3.6
pFE307	15	43	2.9	0.4
pFE309	49	74	1.5	0.7
pFE311	120	48	0.4	2.5
pFE313	180	57	0.3	3.1
pFE316	140	130	0.9	1.1
pFE318	310	73	0.2	4.3
pFE321	290	55	0.2	5.2
pFE326	61	43	0.7	1.4
pFE327	190	98	0.5	2.0
pFE330	38	38	1.0	1.0
pFE332	77	27	0.4	2.8
pFE333	480	86	0.2	5.6
pFE341	15	5	0.3	3.1
pFE342	15	6	0.4	2.6
pFE345	56	20	0.4	2.8
pFE349	29	78	2.7	0.4
pFE353	200	15	0.08	13.2
pFE354	120	81	0.7	1.5
pFE355	120	50	0.4	2.3
pFE357	400	460	1.1	0.9
pFE358	47	19	0.4	2.5
pFE359	20	12	0.6	1.6
pFE360	17	15	0.9	1.1
pFE361	120	65	0.6	1.8
pFE364	190	29	0.2	6.6
pFE375	37	18	0.5	2.0
pFE377	36	16	0.4	2.3
pFE378	95	42	0.4	2.3
pFE379	88	64	0.7	1.4
pFE380	89	31	0.4	2.9
pFE381	21	13	0.6	1.6
pFE382	15	7	0.5	2.1
pFE383	25	54	2.2	0.5

a. Cell were grown for 6 hours in LBE-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10µl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. Aerobic growth was achieved by shaking the culture vigorously rolling drum.

b. ß-galactosidase activity is expressed as nmoles of ONP produced per min per A_{650}.

Table 6. Anaerobic ß-galactosidase specific activity of promoter clones in pFZY1 from E. coli K12.

anaerobic ß-galactosidase sp act[a]	no. of transformants	no. of anaerobiosis -inducible promoter (fold increase)[b]
0-100	78	6 (3)
100-200	9	1 (2)
200-300	2	0
300-500	1	0
500-1500	2	2 (10)

a. Cultures were grown for 6 hours in LB-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10μl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per A_{650}.

b. Fold increase was calculated from the ratio of anaerobic ß-galactosidase sp act/aerobic ß-galactosidase sp act.

Table 7. Aerobic ß-galactosidase specific activity of promoter clones in plasmid pFZY1 from E. coli K12.

Aerobic ß-galactosidase sp act[a]	no. of transformants	no. of aerobiosis inducible promoters (fold increase)[b]
0-100	48	1 (7)
100-200	28	5 (5,6,6,8,13)
200-300	6	2 (5,7)
300-500	7	2 (5,12)
500-1500	3	3 (5,8,12)

a. Cultures were grown for 6 hours in L Broth + ampicillin (50μg/ml) and harvested for ß-galactosidase assay. Aerobic growth condition was achieved by inoculating 10μl overnight culture in 1 ml L broth + ampicillin (50μg/ml) in 20mmX160mm tubes and incubated at 37°C with agitation. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per A_{650}.

b. Fold increase was calculated from the ratio of aerobic ß-galactosidase sp act/anaerobic ß-galactosidase sp act. Fold increase for each clones were listed in the bracket.

Table 8. Aerobic and anaerobic ß-galactosidase specific activities of Escherichia coli strain MC1061-5 with recombinant plasmid pFE29 or pFE117.

plasmid	Sise of DNA insert (kb)	ß-galactosidase sp act[a]		Induction ratio ($-O_2/+O_2$)
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	
pFE29	1.6	160	1600	10
pFE117	2.9	120	1100	9.2

a. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per OD_{650}.

2. Use of plasmid pFZY1 for cloning S. typhimurium anaerobiosis-inducible promoters.

In order to obtain smaller DNA fragment size with anaerobiosis-inducible promoters, chromosomal DNA of S. typhimurium was digested with restriction enzyme Sau3A to generate DNA fragments with sizes of about 0.5 kb. The DNA fragments were dephosphorylated and ligated to BamH1 digested plasmid pFZY1. Totally, 65 clones with anaerobically expressed ß-galactosidase activity were isolated. Their ß-galactosidase activity after aerobic and anaerobic growth were assayed (Table 9) and analysed. Results are listed in Table 10 and Table 11. Among these 65 clones, 12 clones had higher aerobic ß-galactosidase activity than anaerobic activity with greater than 2-fold increase, 8 clones had higher anaerobic ß-galactosidase activity than aerobic activity with greater than 2-fold, and the rest (45 clones) had similar aerobic and anaerobic ß-galactosidase activities. Three of the clones had anaerobic induction ratio (anaerobic activity/aerobic activity) greater than six. Their aerobic and anaerobic activity was shown in Table 12.

Table 9. ß-galactosidase specific activity for Escherichia coli strain MC1061-5 carrying recombinant pFZY1 plasmids containing promoters from Salmonella typhimurium LT2.

Plasmid	ß-Galactosidase sp act[b]		Fold induction	
	Aerobic(+O_2)[a]	Anaerobic(-O_2)	-O_2/+O_2	+O_2/-O_2
pFS1	66	450	6.7	0.1
pFS5	450	560	1.3	0.8
pFS10	110	150	1.3	0.8
pFS11	60	98	1.6	0.6
pFS13	190	190	1.0	1.0
pFS16	700	310	0.4	2.3
pFS18	180	120	0.7	1.5
pFS22	8	220	29.0	0.0
pFS24	19	29	1.5	0.7
pFS26	20	21	1.1	0.9
pFS29	320	4	0.0	82.0
pFS31	340	400	1.2	0.8
pFS33	660	420	0.6	1.6
pFS34	67	460	6.8	0.1
pFS38	340	320	0.9	1.1
pFSs1	200	160	0.8	1.3
pFSs2	110	130	1.1	0.9
pFSs3	1600	1400	0.8	1.2
pFSs4	13	11	0.9	1.1
pFSs5	1300	2800	2.1	0.5
pFSs6	170	76	0.5	2.2
pFSs7	74	110	1.5	0.7
pFSs8	290	120	0.4	2.5
pFS2.1	19	40	2.1	0.5
pFS2.2	40	44	1.1	0.9
pFS2.3	11	24	2.2	0.5
pFS2.4	290	290	1.0	1.0
pFS2.5	91	73	0.8	1.2
pFS2.6	16	74	4.5	0.2
pFS2.7	640	430	0.7	1.5
pFS2.8	76	74	1.0	1.0
pFS2.9	440	260	0.6	1.7
pFS3.0	410	340	0.8	1.2
pFS3.1	36	23	0.6	1.5
pFS3.2	84	27	0.3	3.2
pFS3.3	430	230	0.5	1.8
pFS3.4	37	21	0.6	1.8
pFS3.5	22	27	1.2	0.8
pFS3.6	52	75	1.5	0.7
pFS3.7	120	59	0.5	2.1
pFS3.8	360	170	0.5	2.1
pFS3.9	110	70	0.6	1.6
pFS4.0	69	43	0.6	1.6
pFS4.1	170	180	1.0	1.0
pFS4.2	48	63	1.3	0.8

Table 9 cont'd

pFS4.3	150	260	1.8	0.6
pFS4.4	270	130	0.5	2.0
pFS4.5	190	170	0.9	1.1
pFS4.6	230	340	1.5	0.7
pFS4.7	110	59	0.6	1.8
pFS4.8	61	5	0.1	13.1
pFS4.9	73	50	0.7	1.5
pFS5.0	370	240	0.6	1.6
pFS5.1	150	140	0.9	1.1
pFS5.2	62	29	0.5	2.1
pFS5.3	240	190	0.8	1.3
pFS5.4	41	29	0.7	1.4
pFS5.5	180	68	0.4	2.6
pFS5.6	420	350	0.8	1.2
pFS5.7	91	250	2.8	0.4
pFS5.8	77	27	0.4	2.9
pFS5.9	21	22	1.0	1.0
pFS6.0	140	110	0.8	1.3
pFS6.1	100	58	0.6	1.7
pFS6.2	23	24	1.0	1.0

a. Cell were grown for 6 hours in LBE-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10µl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. Aerobic growth was achieved by shaking the culture vigorously rolling drum.

b. ß-galactosidase activity is expressed as nmoles of ONP produced per min per A_{650}.

Table 10. Anaerobic ß-galactosidase specific activity of Salmonella typhimurium promoter clones with recombinant plasmid pFZY1.

Anaerobic ß-galactosidase sp act[a]	no. of transformants	no. of anaerobiosis -inducible promoter (fold increase)[b]
0-100	31	3 (2, 2, 5)
100-200	14	0
200-300	7	2 (3, 29)
300-400	5	0
400-500	5	2 (7,7)
500-1000	1	0
1000-2000	1	0
2000-3000	1	1 (2)

a. Cultures were grown for 6 hours in LB-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10μl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. ß-galactosidase specific activity is expressed as nmoles of o-nitrophenol produced per min per A_{650}.

b. Fold increase was calculated from the ratio of anaerobic ß-galactosidase sp act/aerobic ß-galactosidase sp act.

Table 11. Aerobic ß-galactosidase specific activity of Salmonella typhimurium promoter clones with recombinant plasmid pFZY1.

Aerobic ß-galactosidase sp act[a]	no. of transformants	no. of aerobiosis inducible promoter (fold increase)[b]
0-100	29	4 (2, 3, 3, 13)
100-200	15	3 (2, 2, 3)
200-300	6	2 (2, 3)
300-400	5	2 (2, 81)
400-500	5	0
500-1000	3	1 (2)
1000-2000	2	0

a. Cultures were grown for 6 hours in LB-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10μl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. ß-galactosidase specific activity is expressed as nmoles of o-nitrophenol produced per min per A_{650}.

b. Fold increase was calculated from the ratio of anaerobic ß-galactosidase sp act/aerobic ß-galactosidase sp act.

Table 12. Aerobic and anaerobic ß-galactosidase activities of Escherichia coli strain MC1061-5 with recombinant plasmid pFS1, pFS22 or pFS34.

plasmid[a]	Size of DNA insert (kb)	ß-galactosidase sp act[b]		Induction ratio ($-O_2/+_2$)
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	
pFS1	0.6	66	450	6.8
pFS22	0.5	8	220	28
pFS34	0.6	67	460	6.9

a. pFS1, pFS22 and pFS34 contained DNA fragments from Salmonella typhimurium LT2.

b. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per OD_{650}.

Since the preparation of large amount of low-copy-number plasmid DNA was tedious, polymerase chain reaction (PCR) (Saiki et al., 1988) (discussed in chapter 3) was used to amplify the cloned DNA fragments for size determination and for later subcloning. The DNA sequences at both sides of the cloning sites of plasmid pFZY1 were determined (discussed in Chapter 3) and two primers of sequences shown below were used for PCR reaction :-

1. pBR322 EcoR1 clockwise extended primer (EcoR1-ext):

   5'-TAGGCGTATCACGAGGCCCT-3' (20 mer)

2. galK Leader primer (54 bases from the HindIII site of pFZY1) [galK(-54)]:

   5'-TACGGTGGCGGAGCGCAGCA-3'(20 mer)

Figure 6 showed that DNA fragments generated by PCR reaction from plasmids pFS1, pFS22, and pFS34, which were about 0.6 kb, 0.5 kb and 0.6 kb respectively.

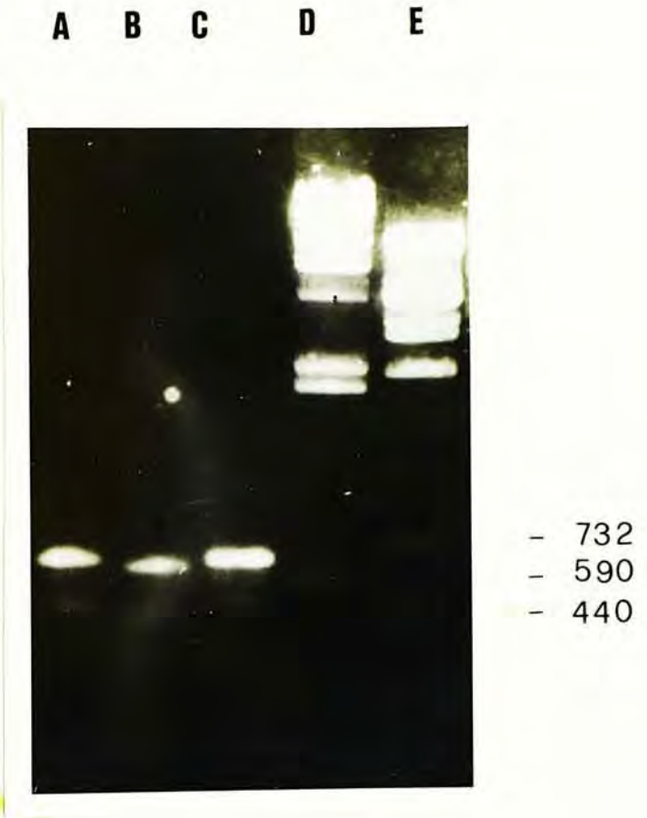


Figure 6. Gel electrophoresis of PCR fragments generated from pFS1, pFS22 and pFS34 with primers EcoR1-ext and galK(-54). Lanes: A, pFS1; B, pFS22; C, pFS34; D, HindIII-digested Lambda standards; E, HpaI digested Lambda standards. DNA samples were analysed in 1.2% agarose gel. The direction of electrophoresis was from top to bottom. The sizes of standards were indicated in base pairs.

IV. Summary and Discussion

A "shot gun" approach was employed for the cloning of anaerobiosis-inducible promoters from E. coli and S. typhimurium. Two promoter probe plasmids were used - pKK232.8 and pFZY1. These promoter probe plasmid systems generate transcriptional fusion for all promoters cloned. Both of them gave positive results, but the use of pFZY1 was relatively easy and accurate. The following paragraphs disscused the cloning with plasmid pKK232.8 and pFZY1 used in this study.

A. Cloning with promoter-probe plasmid pKK232.8.

With the promoter probe plasmid pKK232.8, 6 clones with anaerobiosis-inducible CAT activity were identified after screening for their resistance to chloramphenicol of 150μg/ml under aerobic and anaerobic condition. The advantage of plasmid pKK232.8 is its positive selection procedure. Any transformants which are resistant to chloramphenicol are likely to have recombinant plasmid pKK232.8 with promoter containing DNA fragment cloned. Moreover, the level of resistance to chloramphenicol can reflect the promoter activity. That is, the higher level of chloramphenicol resistance under anaerobic condition than that of aerobic condition would indicate that the clone has a recombinant plasmid with anaerobiosis-inducible promoter. However, pKK232.8 is a high-copy-number plasmid. The CAT activity would not really reflect the expression of the

promoter in the chromosome condition. The regulation of the promoter activity would be affected by the copy number of the plasmid under different conditon tested. Moreover, if a cloned anaerobiosis promoter has a significant aerobic activity, promoter would be regarded as constitutive. That is, these clones would be resistant to chloramphenicol (150μg/ml) at both aerobic and anaerobic conditions. As a result, some anaerobiosis-inducible promoter clones would be missed during the screening procedure. Another problem with the screening precedure was the anaerobic growth condition. Since anaerobic growth of E. coli in LB plate is poor, only tiny colonies were observed after 24 hours of growth. Thus, LGE plates with various chloramphenicol concentrations were used. The growth of E. coli was greatly improved and comparable to that of aerobic growth. The addition of glucose in the agar plate can repress the expression of some anaerobiosis-inducbile promoters (Winkelman and Clark , 1986). This may account for the small number of anaerobiosis promoter clones (only six) obtained from 1132 promoter clones.

B. Cloning anaerobiosis-inducible promoters with pFZY1 plasmid.

With the promoter probe plasmid pFZY1, promoter clones were constructed for both E. coli and S. typhimurium. Nine clones with anaerobiosis-inducible β-galactosidase activity were identified from 92 E. coli anaerobic expressed promoter clones. Eight clones with anaerobiosis-inducible

ß-galactosidase activity were identified from 65 S. typhimurium anaerobic expressed promoter clones.

Koop et al. (1987) demonstrated that the regulation of the wild-type lac promoter and mutants in pFZY1 was similar to that observed for lac promoters in the chromosome. Since the assay of ß-galactosidase activity is easy, the ß-galactosidase activity of all anaerobic expressed promoter clones were determined with cells grown under aerobic or anaerobic condition. This screening procedure is quantitative. As a result, all anaerobiosis-inducible promoter clones with higher anaerobic acitivty would be identified. pFZY1 is a low-copy-number plasmid with 1-2 copy per genome (Koop et al., 1987); thus, the regulation of the promoters cloned would not be affected by the copy number of the plasmid. The regulation of promoters cloned in pFZY1 would be similar to that in the chromosome.

The disadvantage of using pFZY1 is also related to its low-copy-number. To test for the size of DNA insert, small scale preparations of recombinant plasmid was done by the alkaline-SDS precedure of Birnboim (1983). However, the amount of plasmid prepared was too little to be analysed, especially when the size of insert was smaller than 1 kb. However, this problem was overcome by the using of PCR reaction to amplify the DNA insert from small scale prepared plasmids for analysis.

C. Number of anaerobiosis inducible promoters

As estimated by Clark (1984), the number of anaerobiosis-inducible genes is about 50. Over 1,000 genes have been identified in E. coli K12 (Bachmann, 1983) and over 1,800 polypeptides are resolved from extracts of any given E. coli culture on O'Farrell two-dimensional gels (O'Farrell et al., 1977). Thus if we assume there are 2,000 genes in E. coli, the percentage of anaerobic inducible genes would be 2.5% (50/2000). That is for every 100 promoters screened, there is a possibility of obtaining 2.5 anaerobic inducible promoters. Results in the present study with pFZY1 cloning system for anaerobiosis-inducible promoters were of higher percentage, 9.8% (9/92) for E. coli and 12.3% (8/65) for S. typhimurium. The higher percentage would be due to the screening procedure used. Only clones which form blue colonies on X-gal plate under anaerobic conditions (i.e. anaerobic expressed promoters) were subjected to ß-galactosidase assay.

To conclude, the "shot gun" approach for cloning anaerobiosis-inducible promoter was successful. The system with low-copy-number promoter probe plasmid pFZY1 is preferable. Coupling with PCR reaction to analyze cloned promoters, the cloning strategy with pFZY1 is a good system and will be useful for the cloning of other global regulated promoters.

Chapter 3

Subcloning and Sequencing

I. Introduction

Several anaerobiosis-inducible promoters containing DNA fragments were cloned as discussed in Chapter 2. In order to trim down the size of fragments which were likely to contain the anaerobiosis-inducible promoters, the restriction map of larger DNA fragments were determined and used to subcloned small fragments. Another reasons for subcloning is to obtain sequence data. The anaerobiosis-inducible promoters cloned have different anaerobic induction ratios, and those with higher anaerobic induction ratios were subjected to subcloning, sequencing, and further studies. The subcloning and sequencing of anaerobiosis-promoter-containing plasmids pFE29 and pFE117 of Escherichia coli , and pHSK1, pHSK8, pFS1, pFS22, and pFS34 of Salmonella typhimurium were done. For sequencing, the enzymatic method of Sanger et al. (1977) was used throughout the study using single-stranded DNA templates prepared form M13mp phage subclones or double-stranded templates from CsCl-EtBr centrifugation purified recombinant plasmid pFZY1 clones.

II. Materials and Methods

A. Bacterial strains and bacteriophages. Escherichia coli strain JM101 was used as the host for propagation of M13mp18/19 phages. For transformation, Escherichia coli

strain MC1061-5 was used for M13 mp18/19 RF plasmids, but JM101 for M13mp9.

B. Preparation of M13mp RF plasmid. The procedure used was that of alkaline-SDS lysis method (Birnborm, 1983). The cell harvested was washed with SET buffer to remove single stranded phages in the broth and the procedure was followed as described in Chapter 2.

C. DNA sequencing by the chain termination method (Sanger et al., 1977).

1. Preparation of single stranded template from recombinant M13 phage.

Twenty µl overnight culture of JM101 culture was added to 2 ml of 2YT medium. A single plaque of the recombinant M13mp phage or 20 µl M13 phage stock was picked with a sterile Pasteur pipette and transferred to the 2 ml diluted JM101 culture in a 100 X 16 mm sterile test tube. The tube was shaken at 37°C for 6-8 hours. The grown culture was centrifuged in an Eppendorf centrifuge for 5 minutes at 4°C. One ml of the supernatant was carefully transferred to another Eppendorf centrifuge. 250µl of 2.5M NaCl, 20% PEG 6000 was added, mixed well and kept at ice for 15 minutes. The mixture was centrifuged in an Eppendorf centrifuge for 10 minutes at 4°C. Supernatant was carefully removed. The PEG pellet was visible and was resuspended in 100µl TE. It was extracted with 100µl neutralized phenol. The aqueous phase was transferred to another Eppendorf tube. The phenol

phase was back extracted with 100µl TE and the aqueous phase was pooled together. To 200µl aqueous phase, 20µl 3M sodium acetate, pH5.2, and 450µl absolute ethanol were added and mixed well. The mixture was kept in -20°C overnight or -70°C for 1 hour, and then centrifuged in eppendorf centrifuge for 15 minutes at 4°C. The pellet was washed with cold 80% ethanol and dried under vacuum. It was resuspended in 30µl sterile double distillated water and 10µl was used for each sequencing reaction or kept in -20°C for later used.

2. Preparation of 6% acrylamide sequencing gel.

The acrylamide gels were set up in the Sequi-Gen Nucleic acid Sequencing System (Bio-Rad). Before setting the gel apparatus, clean glass plates were washed with absolute ethanol and dried. The glass plate of the Integral Plate/Chamber(IPC) was treated with Sigmacote (siliconized solution, Sigma) which was spread evenly on the clean IPC glass plate with Kimwipe tissue. The glass plate was allowed to dry for 5 minutes. The glass plates were then assembled according to the instruction manual of Manufacturer with wedge spacers. For a 50 X 21 cm gel with wedge spacers (0.25-0.7mm), 70 ml urea-acrylamide mix was used. 10X TBE buffer, 20% acrylamide solution (A:B, 20:1) in 46% urea, and 46% urea stock solution were prepared first. For a 6% acrylamide gel, 7ml 10xTBE, 21ml 20% acrylamide solution, and 42ml 46% urea were mixed together. To the urea-acrylamide mix, 0.1g ammonium persulfate was added and dissolved and the mix was filtered through a millipore

filter (Schleicher and Schuell, 0.45 mm). Fifty µl TEMED was added to 15 ml urea-acrylamide mix which was used immediately to seal the base of the assembled glass plates in the casting tray. The rest of the urea-acrylamide mix was cooled in ice. After the base of the assembled glass plates was sealed, 10 µl TEMED was added to the cooled urea-acrylamide mix (about 55 ml) and immediately dispensed into the cavity between the two glass plates using a 50 ml syringe. The gel was allowed to set for 2 hours before used. Alternatively, the gel could be set a day before the DNA sequencing experiment.

3. Sequencing reaction using single-stranded templates.

The reagents used in the sequencing reaction were T7 polymerase sequencing Kit from Pharmacia and the procedure followed the instruction manual.

(i) Annealing

Ten µl M13 template (1-2µg), 2µl annealing buffer, and 2µl sequencing primer (5mM) were mixed well in an Eppendorf tube. The Eppendorf tube was heated in 65°C thermal block for 10 minutes and then cooled back to room temperature.

(ii)Labeling reaction

To the tube containing the annealed template and primer, 3µl Labeling mix, 1µl(10µCi) [α-^{35}S]dATPαS, and 2µl(3 units) diluted T7 DNA polymerase were added. The labeling mix was incubated at room temperature for 5 minutes.

(iii)Termination reaction

After the Labeling mix has been incubated for 5 minutes, 4.5 µl of this reaction was transferred into each of four pre-warmed sequencing mixes. The mixture was incubated at 37°C for 5 minutes. At the end of 5 minutes, 5µl stop solution was added to each tube. The components were mixed and spinned down with microcentrifuge.

4. Sequencing reaction for double-stranded plasmid

The procedure was that of Chen and Seeburg (1985). Plasmid DNA was denatured first and the reactions followed. Plasmid DNA prepared from CsCl-EtBr centrifugation gradient was used. The concentration of the double stranded plasmid was adjusted to about 2µg in 8µl water. To the 8 µl template, 2µl 2M NaOH was added and incubated at room temperature for 10 minutes. Then, 3µl 3M sodium acetate (pH4.8) 7µl distilled water, and 60µl ethanol were added, and mixed. The mixture was placed in -70°C for one hour. The precipitated DNA was collected by centrifugation and washed with 80% ice-cold ethanol. The DNA pellet was than dried under vacuum and resuspended in 10µl distilled water. One µl primer solution (20µM) and 2 µl annealing buffer was added to the 10µl denatured plasmid. The mixture in Eppendorf tube was placed at 37°C for 20 minutes to anneal the primer. Then the annealed mixture was left at room temperature for 10 minutes before performing the sequencing reaction. Labeling reaction and termination reaction were then performed as described for single-stranded template above.

5. Electrophoresis of the samples

The polyacrylamide gel was run at 1500V for about 30 minutes until the temperature was about 50°C. Then a shark-tooth comb was inserted between the glass plates to form wells. The samples after termination reaction were heated at 80°C for 2 minutes. 3µl heated sample was loaded into the appropriate well of a sequencing gel. The gel was run at 1800V for 3 hours. For second loading of samples, the sample was loaded when the dye xylene cyanol of first loaded sample had reached the bottom of the gel.

D. Polymerase chain reaction (PCR) for the amplification of DNA fragments cloned in plasmid pFZY1. The reaction was performed in 100µl volume containing 10µl template (1-100ng), 5µl of each two primer solutions (20µM), 10µl 10x reaction buffer, 16µl dNTPs (1.25mM each dNTP) and 0.5µl Taq DNA polymerase (2.5 units). The reagents were purchased from Perkin Elmer-Cetus, Norwalk, U.S.A. as a GeneAmp^{TM} DNA Amplification Reagent Kit. Before adding Taq DNA polymerase, the reaction mixture was heated at 95°C for 5 minutes to inactivate any protease which might be present. To prevent evaporation during the reaction cycle, 100µl mineral oil was added to cover the reaction mixture. The PCR was carried out in thermal blocks for 25 cycles (each cycle consisting of 1 minute at 95°C, 1 minute at 45°C and 3 minutes at 72°C), followed by a 10-minutes incubation at 72°C. After the reaction, mineral oil was removed with chloroform. The oligonucleotide primers used for PCR were:

1. pBR322 EcoR1 clockwise extended primer (EcoR1-ext):

5'-TAGGCGTATCACGAGGCCCT-3' (20 mer)

2. galK Leader primer (54 bases from the HindIII site of pFZY1) [glak(-53)]:

5'-TACGGTGGCGGAGCGCAGCA-3'(20 mer)

E. Using Exonuclease III to construct unidirectional deletions to generate nested clones. A 1.9 Kb Eco RI - Sal I fragment was subcloned from pFSH8 into M13mp18. RF plasmid from the subclone was prepared and purified by CsCl-EtBr centrifugation gradient. The purified RF plasmid was digested completely by restriction enzyme Sal I and Pst I, leaving 5' and 3'-overhangs. To 12.5µl linearized DNA (2.5 µg), 5 µl 5x exonuclease III buffer and 6.3 µl water was added. The mixture was prewarmed at 37°C and then 1.2 µl exonuclease III (120U) was added. Three µl reaction mixture was removed at 1 minute interval. The removed sample was heat-treated at 70°C for 10 minutes to stop exo III digestion. 15 µl S1 nuclease buffer, 3 µl water, and 4 µl (4U) S1 nuclease were added to the 3 µl sample. S1 digestion was allowed for 20 minutes at room temperature. After S1 digestion, 8 µl sample was taken out for analysis by agarose gel electrophoresis. To the remaining sample, 1 µl dNTPs mix (0.25mM for each dNTP) and 1 µl (2U) DNA polymerase I, Klenow fragment was added to create blunt ends. Then 13 µl exo III ligation buffer, 2 µl 10mM ATP and 1µl (1U) T4 DNA ligase were added to recircularize the deleted molecules. The recircularized RF plasmid was used to transform

electro-competent cells MC1061-5. The transformed cells MC1061-5 was mixed with 200µl overnight culture of JM101 for M13 propagation to form plaques on soft agar overlay. The nested clones were analyzed for their sizes by direct gel electrophoresis.

F. Direct gel electrophoresis. Single M13 phage was picked with a Pasteur pipette and transferred to 1.5ml 2YT medium and grown for 6 hours. Cells were removed by centrifugation in Eppendorf tube and supernatant (the M13 phage solution) was transferred to a new Eppendorf tube. To 20µl M13 phage solution, 2µl 2%SDS was added and the mixture was incubated at 65°C for 20 minutes. Then 4 µl loading buffer was added and the sample was loaded into the well of 1% agarose gel. After electrophoresis, the single stranded-DNA was stained with ethidium bromide.

G. C-test: screening for the orientation of insert in M13 phage. Recombinant M13 phages to be tested was annealed to a recombinant M13 phage with known insert orientation. Ten µl phage solution from each recombinant phages were mixed and 2µl 2% SDS was added to the mixture. The mixture was incubated at 65°C for 20 minutes. After the incubation, 1µl 5M NaCl was added and left in room temperature for 2 minutes. Four µl loading buffer was added and the sample was loaded into agarose gel for electrophoresis. For phages with different orientation of same insert DNA, the phage DNAs would form a figure eight and moved slower in the gel.

III.Results

A. Subcloning and sequencing of pFE29 and pFE117

The DNA insert size of pFE29 and pFE117 were 1.6kb and 2.9kb respectively as discussed in Chapter 2. pFE29 was digested with restriction enzymes EcoR1 and Sal1 to generate a 1.6 kb EcoR1-Sal1 fragment for forced cloning into M13mp18/19 RF. pFE117 was digested with restriction enyzme EcoR1 and HindIII to generate a 2.9 kb EcoR1-HindIII fragment. The fragments were ligated to EcoR1 and Sal1 or EcoR1 and HindIII double digested and dephosphorylated M13mp18 and M13mp19 RF plasmid. Single-stranded DNA was prepared from the recombinant M13 phages for sequencing. M13 universal primer (5'-GTTTTCCCAGTCACGAC-3') was used for the sequencing reaction. About 200 bp sequence data were read from the sequence latter on the X-ray film. Since the orientation of the fragments in M13mp18 was opposite to that M13mp19, DNA sequences (about 200np) from both ends of the fragments cloned from pFE29 and pFE117 were determined using the M13 universal primer. The sequence data were shown in Figure 7 and Figure 8.

EcoR1

5'-GAATTCGAGCTCGGTACCCGGGGATCGGCAGTCGTACAGGGGCGCGTGTT

GCCCAGTTTGCGGCATACCGCGTCGGTAGCCCATTCAGCCATCAGCCGAT

AGGTCATCAGTTTGCCACCGGTGATGGTGATAAATCCGTCCAGACCATCG

CGTTCAGCATGGTCGAGCAGCACGATGCC.....

.....CGTTACCTGCCGCGGAGATCGATCCGACTTATCG

TCGATTGCGCTGGCAAATTTTCCTGGGGATATTCTTTGGCTATGCGGCTT

ACTATTTGGTTCGTAAGAACTTTGCGCTTGCTATGCCTTATCTGGTTGAG

CAGGGATTCTCACGCGGTGATTTAGGTTTTGCCCTTTCGGGGATCCTCTA

GAGTCGAC-3'

SalI

Figure 7. Sequence from both ends of the 1.6 kb EcoR1-Sal1 fragment of pFE29.

EcoR1
GAATTCGAGCTCGGTACCCGGGGATCAACCTTTACCGCCATCAGCATGGT

TGCGGGGCAGAACTGCAAAGCTGGTTTGAACCTCCAAAACCGGGAACAAC

AAACCTGAGAAGAAGACACCGCCTCCGTT.....

.....GAGAAAGAACGTGGCGGCATTGTGTCAGTGTGGA

ACTGTGCGCACAACGTCGGTGGTGGTATTCCGCCGCTGCTGTTCCTGCTG

GGGATGGCCTGGTTCAATGACTGGCATGCGGCGCTCTATATGCCTGCTTT

CTGCGCCATTCTGGTGGCATTATTCGCCTTTGCGATGATGCGCGATACCC

CGCAATCCTGTGGCTTGCCGCCGATCCTCTAGAGTCGACCTGCAGGCATG

CAAGCTT
HindIII

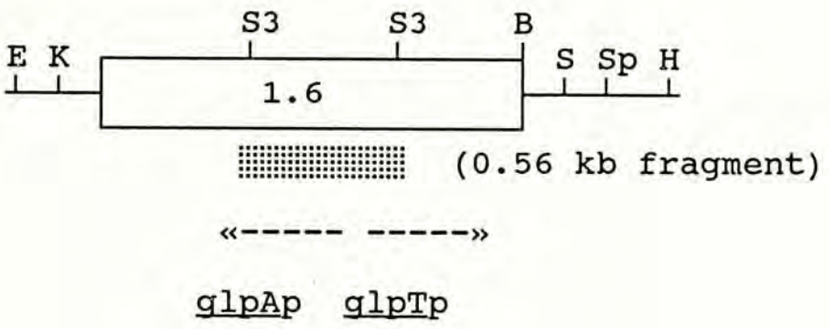
Figure 8. Sequence from both ends of the 2.9 kb EcoR1-HindIII fragment from pFE117.

The DNA sequence from the Sal1 ends of the 1.6kb EcoR1-Sal1 fragment from pFE29 and the DNA sequence from the HindIII end of 2.9 kb EcoR1-HindIII fragment from pFE117 were used for homology search in the GenBank and EMBL database with the FASTA-MAIL program in BIONET system. From the search, both fragment were located in the sequence of glpT coding for glycerol-3-phosphate permease. The Sal1 end of pFE29 fragment corresponded to the 385 bp from the transcriptional initiation site of glpT. The HindIII end of pFE117 corresponded to the 829bp from the transcriptional initiation site of glpT. Incidentally, both pFE29 and pFE117 were likely to contain the same promoter of glpT.

Since both pFE29 and pFE117 were likely to contain the same promoter of glpT, only pFE29 was subjected to subcloning. The 1.6 kb EcoR1-Sal1 fragment from pFE29 was purified by low-melting agarose and digested completely with restriction enzyme Sau3A. The Sau3A products were ligated with BamH1 digested plasmid pFZY1. Recombinant pFZY1 was used to transform competent cell MC1061-5. Blue colonies appeared on X-gal plate with ampicillin were picked and purified.

From the Sau3A digestion of the 1.6 kb EcoR1-Sal1 fragment, one of the Sau3A-Sau3A fragments was expected to be 0.56kb (Figure 9). This 0.56kb Sau3A-Sau3A fragments should contain the promoters of glpAB and glpTQ, which are transcribed divergently (Ehrmann et al., 1987). Hence from the subcloning of these Sau3A-Sau3A fragments, glpT-lac and

pFE29



———— vector sequences with cloning sites

▭ 1.6 kb anaerobiosis-inducible promoter-containing DNA fragment

* only two Sau3A sites were shown here

E (EcoRI), K (KpnI), S (Sal I), H(HindIII), Sp (SphI), S3 (Sau3A), B (BamHI)

Figure 9. The restriction map of 1.6 kb DNA fragment, which contains the promoters of glpA and glpT cloned as pFE29.

glpA-lac were expected to be generated with the pFZY1 plasmid. The purified blue colonies of the transformants in X-gal plate were subjected to ß-galactosidase assay. ß-galactosidase activities were determined with cells grown under aerobic and anaerobic condition for about 8 hours. Two transformants (with plasmid pFE32 and pFE33) with anaerobiosis-inducible ß-galactosidase activities but different activity values (Table 13) were used for large scale preparation of recombinant plasmid. Prepared plasmids were then used as template for sequencing using galK(-54) primer and EcoR1-ext primer.

The nucleotide sequence determined for the lacZ fusion juction of the DNA inserts in pFE32 and pFE33 were shown in Figure 10. From the sequence data of Figure 10, pFE32 had the promoterless lacZ gene expressed by the promoter of glpA and pFE33 had the promoterless lacZ gene expressed by the promoter of glpT. However, the DNA insert in pFE32 was 1.3 kb instead of the 0.56kb, while that of pFE33 was 0.72 kb. The larger size for pFE32 was due to a linear concatamer formation between the 0.56 kb with other Sau3A-Sau3A fragments generated when the 1.6 kb EcoR1-Sal1 fragment was digested with Sau3A. On the other hand, the large size for pFE33 was due to incomplete Sau3A digestion. Nonetheless, glpAB-lac and glpT-lac fusion were generated for expression studies as discussed in Chapter 4.

Table 13. ß-galactosidase specific activity of strain MC1061-5 with plasmid pFE32 or pFE33.

plasmid	Size of DNA insert (kb)	ß-galactosidase sp act[b]		Induction ratio ($-O_2/+O_2$)
		Aerobic[a] ($+O_2$)	Anaerobic ($-O_2$)	
pFE32	0.72	54	580	11
pFE33	1.3	190	1200	6.3

a. Cell were grown for 6 hours in LBE-Amp^{50} and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10µl overnight culture in 1 ml LB-Amp^{50} and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. Aerobic growth was achieved by shaking the culture vigorously rolling drum.

b. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per A_{650}.

glpA-lac fusion in pFE32 (only partial sequence at 5' end shown)

```
5'-.......CAGAGGGATAACAATGAAAACTCGCGACTCGCAATCAAGTGACGTGATTA
                    intiation codon for glpA
          TCATTGGCGGCGGCGCAACGGGAGCCGGGATTGCCCGCGACTGTGCCCTG

          CGCGGGCTGCGCGTGATTTTGGTTGAGCGCCACGACATCGCAACCGGTGC

          CACCGGGCGTAACCACGGCCTGCTGCACAGCGGTGCGCGCTATGCGGTAA

          CCGATGCGGAATCGGCCCGCGAATGCATTAGTGAAAACCAGATCTGCGAT
                                                Sau3A
          TTCGCATGTTCCCGGCAAAGGATC----------» lacZ
                              Sau3A
```

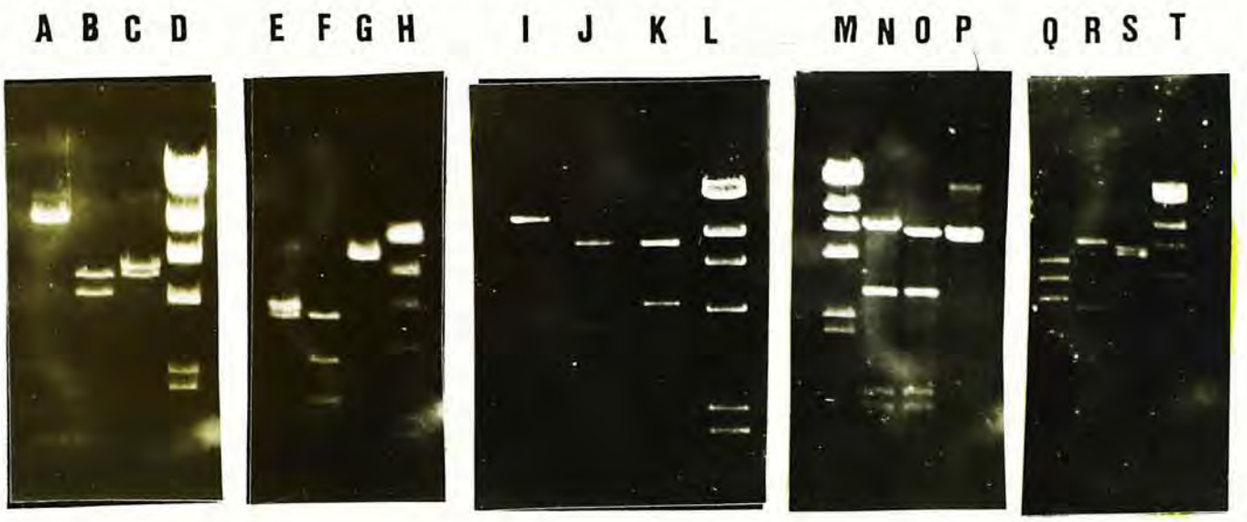
glpT-lac fusion in pFE33 (complete sequence of the DNA insert)

```
          Sau3A
     5'-GATCTGGTTTTCACTAATGCATTCGCGGGCCGATTCCGCATCGGTTACCG

        CATAGCGCGCACCGCTGCAGCAGGCCGTGGTTACGCCCGGTGGCACCGGT

        TGCGATGTCGTGGCGCTCAACCAAAATCACGCGCAGCCCGCGCAGGGCAC

        AGTCGCGGGCAATCCCGGCTCCCGTTGCGCCGCCGCCAATGATAATCACG

        TCACTTGATTGCGAGTCGCGAGTTTTCATTGTTATCCCTCTGAAGTTCGT

        TTTTTACCATTTAGCCATAGTAAAAACATGAATTGTTTGATTTCGCGCAT

        ATTCGCTCATAATTCGAAAGTGAAACGTGATTTCATGCGTCATTTTGAAC

        ATTTTGTAAATCTTATTTAATAATGTGTGCGGCAATTCACATTTAATTTA

        TGAATGTTTTCTTAACATCGCGGCAACTCAAGAAACGGCAGGTTCTCTCA

        CTGAATCAGGCTGTTAATCATAAATAAGACCACGGGCCACGGAGGCTATC

        AATGTTGAGTATTTTTAAACCAGCGCCACACAAAGCGCGCTTACCTGCCG
        initiation codon for glpT
        CGGAGATCGATCCGACTTATCGTCGATTGCGCTGGCAAATTTTCCTGGGG

        ATATTCTTTGGCTATGCGGCTTACTATTTGGTTCGTAAGAACTTTGCGCT

        TGCTATGCCTTATCTGGTTGAGCAGGGATTCTCACGCGGTGATTTAGGTT

        TTGCCCTTTCGGGGATC----------» lacZ
                    Sau3A
```

Figure 10. Nucleotide sequence of DNA insert in pFE32 and pFE33 showing the lacZ junction. The initiation codon for glpA in pFE32 and the initiation codon for glpT in pFE33 were underlined. galK(-54) and EcoR1-ext primers were used in sequencing reactions with double stranded pFE32 and pFE33 DNA templates.

B. Subcloning and sequencing of pHSK1

The DNA insert size of pHSK1 was about 7.1 kb. pHSK1 were digested with various restriction enzymes singly or in different combinations (Figure 11). The restriction map of pHSK1 was shown in Figure 12. A 1.3 kb HindIII-HindIII fragment was subcloned into HindIII digested pKK232.8 plasmid as pHSK11 and M13mp9 RF plasmid. pHSK11 could still confer chloramphenicol resistance to the host harboring it with higher resistance level under anaerobic condition (Table 14). Thus it was expected that the 1.3kb HindIII-HindIII fragment was likely to contain an anaerobiosis-inducible promoter.

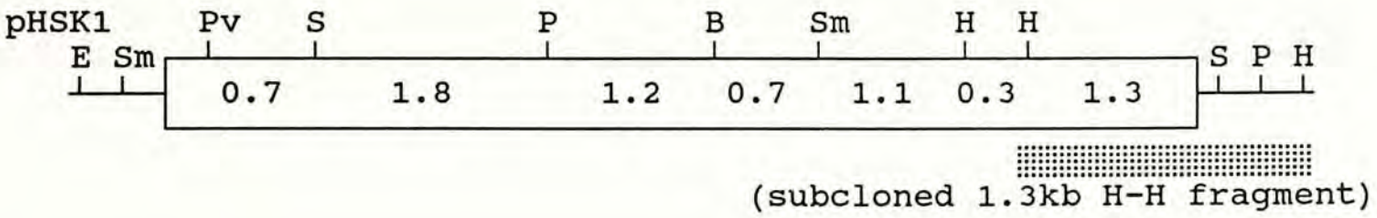


HindIII-digested Lambda standards: Lanes D, H, L, M and T

Restriction enzymes used in each Lanes:

A — HindIII	B — SalI	and	HindIII	C — SalI
E — SalI	F — SalI	and	BamHI	G — BamHI
I — BamHI	J — SmaI	and	BamHI	K — SmaI
N — PvuII	O — PvuII	and	SalI	P — SalI
Q — PstI	R — PvuII			S — SalI

Figure 11. Gel electropheresis of restriction enzyme digested pHSK1. DNA samples were analysed in 0.7% agarose gel. The direction of electrophoresis was from top to bottom. The sizes of standards in base pairs (from top to bottom) were 23670, 9460, 6660, 4260, 2300, and 1960 respectively.



——————— vector sequences with cloning sites

▭ 7.1 kb anaerobiosis-inducible promoter-containing DNA fragment

E (EcoRI), Sm (SmaI), Pv (PvuII), S (SalI), P (PstI), B (BamHI), H (HindIII)

Figure 12. The restriction map of 7.1 kb DNA fragment, which contains an anaerobiosis inducible promoter, cloned as pHSK1.

Table 14. Chloramphenicol acetyltransferase activity of the 1.3 kb DNA fragment subcloned from pHSK1.

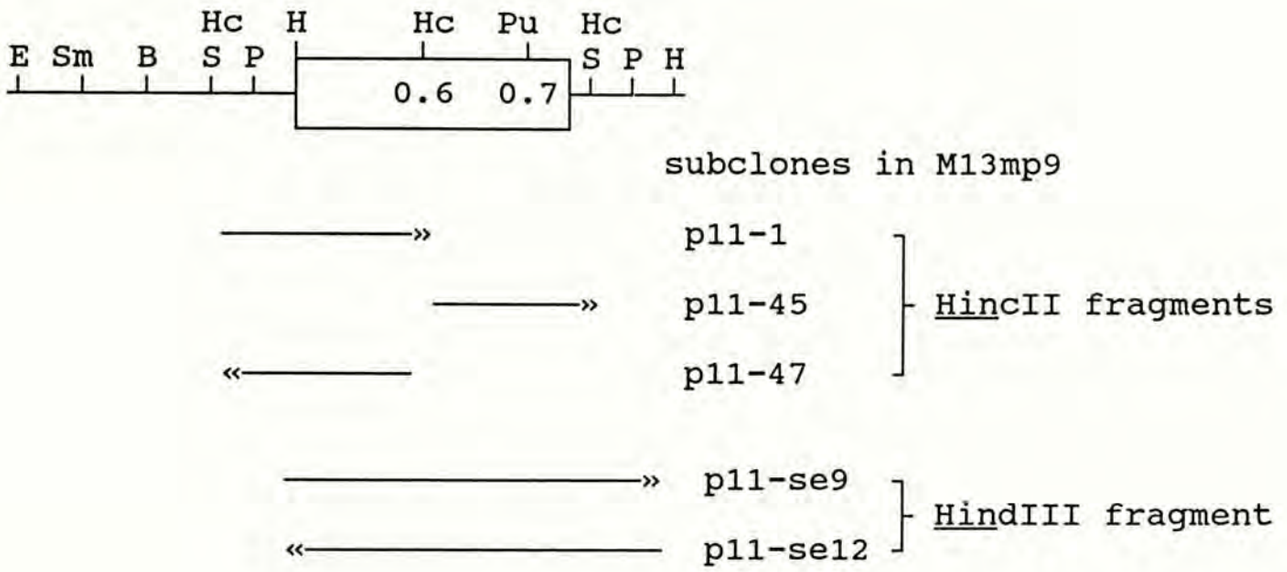
Plasmid[a]	size of insert (kb)	CAT sp act (U/mg protein)[b]		Induction ratio ($-O_2/+O_2$)
		aerobic	anaerobic	
pKK232.8	0	0	0	--
pHSK1	7.1	0.03	1.2	40
pHSK11 (subcloned from pHSK1)	1.3	0.003	0.11	37

a. Plasmid was transformed in E. coli JM107 for assay.

b. One unit of chloramphenicol acetyl transferase (CAT) is defined as 1 µmole of chloramphenicol acetylated per min at 37°C. Cultures were grown in LG-Amp^{50}.

The restriction map of pHSK11 was determined (Figure 13). The complete nucleotide sequence of this 1.3 kb HindIII-HindIII fragment was determined. The sequencing strategy involved cloning specific restriction fragments in M13mp phages. The pHSK11 was digested with restriction enzyme HindIII to generate the 1.3 kb HindIII-HindIII. The orientation of the 1.3 kb HindIII-HindIII fragment in M13mp9 was determined by digesting the RF plasmid prepared from M13mp9 subclones with restriction enzyme Sal1. If a 1.3 kb Sal1-Sal1 fragment was generated, the orientation of the insert in M13 was opposite to that in the plasmid pHSK11. If no 1.3kb fragment was generated, the orientation was the same as that in pHSK11. Two of the M13mp9 subclones, p11-se9 and p11-se12 were determined (Figure 13). Furthermore, the plasmid pHSK11 was digested with restriction enzyme HincII to generate three 0.6kb HincII-HincII fragments, which was then subcloned into M13mp9 for sequencing. The HincII-HincII M13mp 9 subclones was identified by C-test among themselves and with the HindIII-HindIII subclones p11-se9 or p11-se12 (Figure 13 and Figure 14).

pHSK11



——— vector sequences with cloning sites

▭ 1.3 kb anaerobiosis-inducible promoter-containing DNA fragment subcloned from pHSK1

———» size and orientation of subclones of HincII or HindIII fragment from pHSK11

E (EcoRI), Sm (SmaI), S (SalI), P (PstI), B (BamHI), H (Hind III), Hc (HincII), Pu (PvuI)

Figure 13. The restriction map and sequencing strategy of 1.3 kb fragment from pHSK11.

Fig 14a. C-test among HincII-HincII M13mp9 subclones

Lane A-D: HincII-HincII M13mp9 subclones
Lane 1-3: C-test, sample in Lane A with B to D respectively

Lane 3 showed a positive C-test

Fig 14b. C-test among HindIII-HindIII M13mp9 subclones

Lane A-E: HindIII-HindIII M13mp9 subclones
Lane 1-4: C-test, sample in Lane E with A to D respectively

Lane 2, 3 and 4 showed positive C-test

Fig 14c. C-test between a HincII-HincII M13mp9 subclone and HindIII-HindIII M13mp9 subclones

Lane G: HincII-HincII M13mp9 subclone
Lane A-F: HindIII-HindIII M13mp subclones
Lane 1-6: C-test, sample of G with A-F respectively
Lane 2, 3 and 6 showed positive C-test with one additional band moving slower that individual samples

Figure 14. C-test among pHSK11 subclones in M13mp9. C-test was performed by mixing 10 µl of two different M13mp phages. Two µl 2% SDS was then added and the mixture was heated at 65° for 20 minutes. Before loading the test samples, 1µl 5 M NaCl and 4 µl loading buffer was added. Samples were analysed in 1% agarose gel and the direction of electrophoresis was from top to bottom.

The DNA sequence of each subclones was determined and the total DNA sequence of the 1.3 HindIII-HindIII fragment was deduced from the sequences of the M13mp9 subclones. The sequence of the 1.3 kb fragment was given in Figure 15.

Nucleotide sequence of 1350bp HindIII-HindIII fragment in pHSK1

```
          10          20          30          40          50
5' AAGCTTGGCG GCAACACGGC AAGCGCAGTG CCGCCGCGCC AGCGTGTACG
   HindIII
          60          70          80          90         100
   CACAGCATCT CGCCTGAAGA AAACGCCGGG GCGTCTCGAT TTCCAGCGCG

         110         120         130         140         150
   GCATTCTGCA ACGTAGCGCC AACGGCGAAC TGGAGGTCAC GACCACCGGA

         160         170         180         190         200
   CACCAGGGTT CACATATTTT CAGCTCGTTT AGTCTGGGCA ACTGCTTTAT

         210         220         230         240         250
   TGTGCTGGAG CGTGAACGCC GGCAATGTTG AGCCGGGAGA ATGGGTGGAA

         260         270         280         290         300
   GACGAGCCGT TTAATGCTCT GTTCGGAGGC CTGTAATGGC GGAACTTAGC

         310         320         330         340         350
   GACCAGGAGA TCTGCGCTAT AACGACAAAT TATCTGCGCG GCTTCGATTT

         360         370         380         390         400
   GAAGGGCAGG AAGCGTTAAA GATGCGCGGG TATTAGTGGT GCGCTGGAAG

         410         420         430         440         450
   CGTTAAAGAT GCGCGGTATT AGTGGTCGGC TGGCGGCTCG GCTGCGCGGC

         460         470         480         490         500
   AACGCAGTAT CTGGCTGGCG CTGGCGTCGG GCAACTGACG CTACTCGATT

         510         520         530         540         550
   TTGATACCGT TTCCGTTTCC AATCTCCAGC GTCAAACCTT GCACAGCGAC

         560         570         580         590         600
   GCCACGGTCG GGCAGCCGAA GGTAGAGTCC GCCCGCGACG CTGGCGCGTA

         610         620         630         640         650
   TCAACCCACA TATTACCATT ACGCCCGTTA ACGCGCGGCT GGACGACGAC
                                HincII
         660         670         680         690         700
   GCTATGACCA GCCTGATTGC CGGGCATTCG CTGGTGCTGG ACTGTACCGA

         710         720         730         740         750
   TAACGTTAGC GTACGTAATC AACTTAACGC CGGGTGCTAT ACCGCGAAAG

         760         770         780         790         800
   TGCCGCTAAT CTCCGGCGCG GCCATTCGTA TGGAAGGACA AGTTACCGTC

         810         820         830         840         850
   TTTACGTATC GGAAACGACC TGTACGCTGC TGAGCGTCTG TTGCGAAACG
```

Figure 15. cont'd

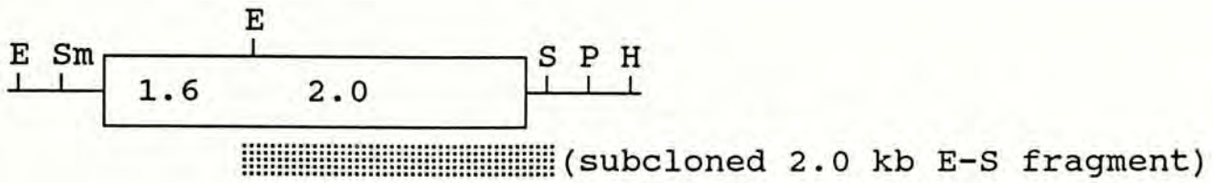
```
        860         870         880         890         900
CTCTGACTGC GTAGAGGCAG GGTGATGGCG CCGCTGATTG GCGTGATCGG

        910         920         930         940         950
TTCGCTACAG GCAATGGAGG CCATTAAGCT ACTGGCGCAT TACGGTCAGC

        960         970         980         990        1000
CTGCCAGCGA AAAATCGTCA TGTACGATGC GATGACCTGT CAGTTCCGCG

       1010        1020        1030        1040        1050
AAATGAAGTT AATGCGCAAC CCCGGCTGTG AGGTCTGCGG GCAGTAGCCA

       1060        1070        1080        1090        1100
CTTTACGGAT AAACATACCA AAGCACGGTT TATGGCCGTG CTGTATCAAC

       1110        1120        1130        1140        1150
AGATAATTAC TGTCATCAGA CGCAAAGCCA GCTTACCCAA GCTCCGACTG

       1160        1170        1180        1190        1200
ACGATCGGCT CAAGCAGCGC GGATCATCTT AAACGCCATA CCACAACCTC
 Pvu1
       1210        1220        1230        1240        1250
AAACCGTGAT GTTGTACCTC ATTTAACGCC TCTTTGTCAG AACCTCTCCA

       1260        1270        1280        1290        1300
TTCGTTGACG CACATCAAGA TAGCTTTCAT TCGAAAGTAA TTTAATCTTT

       1310        1320        1330        1340        1350
ATATGAAATA AGAGAGGCCG TTTATGATCC GTCGACCTGC AGCCAAGCTT 3'
                                  Sal1  Pst1      HindIII
                                  └──────────────────┘
                                           |
                                  cloning site of
                                  vector pKK232.8
```

Figure 15. Nucleotide sequence of 1350 bp of the HindIII-HindIII fragment in pHSK1.

C. Subcloning and sequencing of pHSK8

The restriction map of pHSK8 was determined (Figure 16). It contained a DNA insert of size 3.6 kb. Plasmid pHSK8 was digested with EcoR1 and Sal1 to generate a 2.0 kb EcoR1-Sal1 fragment which was force-cloned in appropriate orientation into pFZY1 by ligating to EcoR1 and Sal1 double-digested pFZY1 plasmid. The resulting recombinant pFZY1 plasmid was named pFSH8. MC1061-5 harboring this plasmid still had anaerobiosis inducible ß-galactosidase activity as shown in Table 15. It indicated that the 2.0 kb EcoR1-Sal1 fragment contained an anaerobiosis-inducible promoter.

pHSK8



——————— vector sequences with cloning sites

▭ 3.6 kb anaerobiosis-inducible promoter-containing DNA fragment

E (EcoRI), Sm (SmaI), S (Sal I), P (PstI), H (HindIII)

Figure 16. The restriction map of 3.6 kb DNA fragment, which contains an anaerobiosis inducible promoter, cloned as pHSK8.

Table 15. ß-galactosidase specific activity of strain MC1061-5 with plasmid pFZH8.

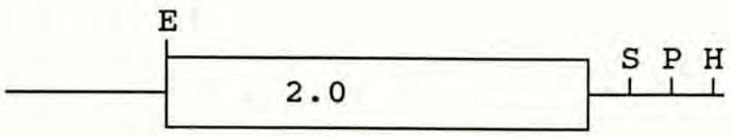
plasmid	Medium	ß-galactosidase sp act[b]		Induction ratio ($-O_2/+O_2$)
		Aerobic[a] ($+O_2$)	Anaerobic ($-O_2$)	
pFSH8	LBE-Amp^{50}	7	64	9
	LBE-Amp^{50} + 0.5% Glucose	8	82	10
	LBE-Amp^{50} + 0.5% Nitrate	5	190	38

a. Cell were grown for 6 hours in the test media and harvested for ß-galactosidase assay. Anaerobic growth condition was achieved by inoculating 10µl overnight culture in 1 ml test media and incubated in anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C as stand culture. Aerobic growth was achieved by shaking the culture vigorously rolling drum.

b. ß-galactosidase specific activity is expressed as nmoles of ONP produced per min per A_{650}.

The nucleotide sequence of the 2.0 kb EcoR1-Sal1 fragement was completely determined. The sequencing strategy was shown in Figure 17. The 2.0 kb EcoR1-Sal1 fragment from pFZH8 was subcloned into M13mp18/19 as p8-1822 1nd p8-1922. RF plasmid was prepared form M13mp18 subclone p8-1822 for the generation of nested deletion subclones by exoIII unidirectional deletion (Figure 17). The size of the nested deletion M13mp18 subclones was estimated by direct gel electrophoresis. A series of nested subclones of different sizes (Figure 18) were chosen for sequencing. The sequence of the 2.0 kb fragment was determined and shown in Figure 19.

pFSH8



subclone in M13mp19
p8-1922

subclone in M13mp18
p8-1822

nested subclones generated from p8-1822 with exoIII

p8-4
p8-O
p8-P
p8-9
p8-15
p8-33
p8-23
p8-8
p8-18
p8-32

——— vector sequences with cloning sites

▭ 2.0 kb anaerobiosis-inducible promoter-containing DNA fragment subcloned from pHSK8

E (EcoRI), S (SalI), P (PstI), H (HindIII)

Figure 17. Restriction map and sequencing strategy of the 2.0 kb fragment from pFSH8.

A B C D E F G H I J K L M N O P Q R S T U V

Lane A and V: parent M13mp subclone p8-1822, no deletion

Lane B and U: M13mp18 with 1.6 kb DNA insert as Marker

Lane C and T: M13mp18, no DNA insert

Lane D to S: Nested M13mp18 subclones , of different DNA insert size, generated by exoIII nuclease unidirectional digestion.

Figure 18. Direct gel analysis of nested M13mp18 subclones containing 2.0 kb EcoR1-SalI fragment from pFSH8.

Nucleotide sequence of 1991 bp EcoR1-Sal1 fragment from pFSH8

```
          10          20          30          40          50
5' GAATTCTGGG CCGATCGCGG CGTGGATGGC CTGCGTCTGG ACGTGGTGAA
   EcoR1
          60          70          80          90         100
   CCTGATCGCC AAAGATCAGA ATTTTCCTGA CGATCCGACG GCGATGGACG

         110         120         130         140         150
   CCGCTTTTAT ACCGACGGGC CGCGCGCGCA TACGTTTTTA CGCGAAATGA

         160         170         180         190         200
   ACCGTGACGT TTTTACGCCG CGTAATCTGA TGACGGTAGG CGAAATGTCC

         210         220         230         240         250
   TCTACCACGC TGGGAAAACT GCCAGCAATA TGCCGCGCTT AGCGGCGACG

         260         270         280         290         300
   AACTCTCGAT GACCTTCAAT TTTCATCATC TGAAGGTGGA TTACCCCAAT

         310         320         330         340         350
   GGCGAAAAGT GGACGCTGGC AAAACCTGAT TATGTGGCGC TGAAAGCGTT

         360         370         380         390         400
   GTTCCGCACT GGCAACAGGG GATGCATAAC GTCGCCTGGA ACGCGTTATT

         410         420         430         440         450
   CTGGTGTAAT CACGATCAGC CGCGCATCTT TCCGCAAAGG CGACGAGGGT

         460         470         480         490         500
   GAATACCGGG TTCCAGCCGC GAAAATGTTG GCGATGGCGC TGCACGGTAT

         510         520         530         540         550
   GCAAGGACGC CTTATATCTA TCAGGGCGAA GAAATCGGCA TGACCAACCC

         560         570         580         590         600
   ACACTTTACC CGCATCACCG ATTATCGCGA CGTGGAAAGC CATAACATGT

         610         620         630         640         650
   TTGCCCGGCT AGCGCGCCGC CGGGCGCGAC CCCGACGAAC TGCTGGCTAT

         660         670         680         690         700
   TCTGGCCAGT AAATCCCGCG ACAACAGCCG CACGCCGATG CAATGGGACA

         710         720         730         740         750
   ACGGTAAAAA CGCCGGTTTC ACCCAAGGCG AGCCGTGGAT AAGCCTGTGC

         760         770         780         790         800
   GATAACTATA CGGAGGTTAA CGTCGAGGCG GCATTACGCG ATGAAAACTC

         810         820         830         840         850
   GGTGTTTTAC ACCTATCAAA AGCTGATTGC GCTACGTAAA ACCCAGCCTG
```

Figure 19. cont'd

```
       860        870        880        890        900
TACTGATCTG GGGCGATTAT CAGGATCTCT CCCGGATAGC CCATCAGTAT

       910        920        930        940        950
GGTGTTATCG CCGCCAGTGG CAGGGCAAAT CCTGCTGGTT GTCGCCAATC

       960        970        980        990       1000
TGAGTAACCA GTGCCAGGAG TGCATCCGCC GCATATCAAA GGACAGTGGC

      1010       1020       1030       1040       1050
AGGCGCTACT GCATAATTAT GCGAGGTCGC CAGCCAGCCA GCGCGATGAC

      1060       1070       1080       1090       1100
GTCCGCCCAT TTGAAGCCAT CTGGTGGTTA CAAGCCTAAC GCGACATCCT

      1110       1120       1130       1140       1150
TATACGGATA AACGCCACAA ACCTATTGAC ACGGGCTGTG GCGTTGCTCC

      1160       1170       1180       1190       1200
AGGTTACTGA CTGTTTTTGT TGAATAATTA ATCAGATTTT TCTTGCTATC

      1210       1220       1230       1240       1250
TTTAACAATG CACAGCGCCG CCATCTGTAG CTCTGATTTT TACCTTGTTC

      1260       1270       1280       1290       1300
TACATCAATA AAATTGCAAA CATCCTTGAT GCAAATCACT ACATATAGAC

      1310       1320       1330       1340       1350
TTTAAAATGC ACACCGACCC AATATGTTGT ATTAATTGAC TACAATTGCT

      1360       1370       1380       1390       1400
ACAACACCTG TTCACTCGAC ACAAGGTGAA TTGTGGATAA CCTGGGTCAG

      1410       1420       1430       1440       1450
GATTGCGGGA AGTCGTTGGA AAAGAGATTG CAATAAAACC TGTTATAGGC

      1460       1470       1480       1490       1500
TTCCCCGGCC TCTGTGGATA ACCTGTTCTT ACAAATATGG AGTGATCATG

      1510       1520       1530       1540       1550
ACACCGCATG TGATGAAACG AGATGGCTGT AAAGTGCCAT TCAAATCAGA

      1560       1570       1580       1590       1600
GCGCATTAAG GAAGCCATTC TACGTGCACG TAAAGCAGCG GGAGTCGATG

      1610       1620       1630       1640       1650
ACGCAGATTA CTGTGCCGTC GCAGAAGTCG TTAGCAGCCA AATGAACGCG

      1660       1670       1680       1690       1700
CGCAGTCAGG TGGATATTAA CGAGATCCAA ACTGCGGTTG AAAACCAACT

      1710       1720       1730       1740       1750
GATGTCCGGC CCGTACAAAC AGCTTGCCCG CGCCTACATC GAATACCGTC
```

Figure 19. cont'd

```
      1760        1770        1780        1790        1800
ACGATCGCGA CATTCAGCGT GAAAAGCGTG GTCGTCTGAA CCAGGAAATT

      1810        1820        1830        1840        1850
CGCGGCCTGG TAGAGCAAAC TAACTCCGCG TTGCTCAATG AAAACGCCAA

      1860        1870        1880        1890        1900
CAAAGACAGT AAAGTCATTC CCACCCAGCG CGATTTGCTG GCCGGGATTG

      1910        1920        1930        1940        1950
TCGCCAAACA CTATGCCCGC CAGCACCTGT TGCCGCGTGA CGTAGTACAC

      1960        1970        1980        1991
ACGCATGAGC GCGGCGATAT CCACTATCAC GATCCGTCGAC 3'
                                       Sal1
```

Figure 19. Nucleotide sequence of 1991 bp EcoR1-Sal1 fragment from pFSH8.

D. Subcloning and sequencing of pFS1, pFS22 and pFS34

The subcloning and sequencing strategy of pFS1, pFS22 and pF34 were shown in Figure 20. DNA inserts in pFS1, pFS22 and pFS34 were generated by PCR reaction using EcoR1-ext and galK(-54) primers. The size of pFS1, pFS22, pFS34 were 0.6, 0.5 and 0.6 kb respectively. The produced PCR DNA fragments were purified with Centricon-30 to remove excess primers and dNTPs. The purified PCR products were digested with EcoR1 and Sal1 and cloned in the appropriate orientation into EcoR1-Sal1-digested M13mp18 RF. Single-stranded DNA template was prepared for sequencing reaction using the M13 universal primer. Alternatively, the purified PCR DNA products were directly ligated to SmaI digested M13mp18 and sequenced with EcoR1-ext or galK(-45) primers. Nucleotide sequences were determined from both ends of the PCR fragments with M13 clones containing DNA inserts in both orientation. The nucleotide sequences from both ends overlapped each other and the total nucleotide sequences were determined for pFS22 and pFS34. Since the M13 subclones from pFS1 had positive C-test with M13 subclones form pFS34. pFS1 and pFS34 were expected to contain similar promoter. Hence, the nucleotide sequence of pFS1 was not determined. The nucleotide sequence of 0.5 kb DNA insert in pFS22 was shown in Figure 21 and the nucleotide sequence of 0.6 kb DNA insert of pFS34 was shown in Figure 22.

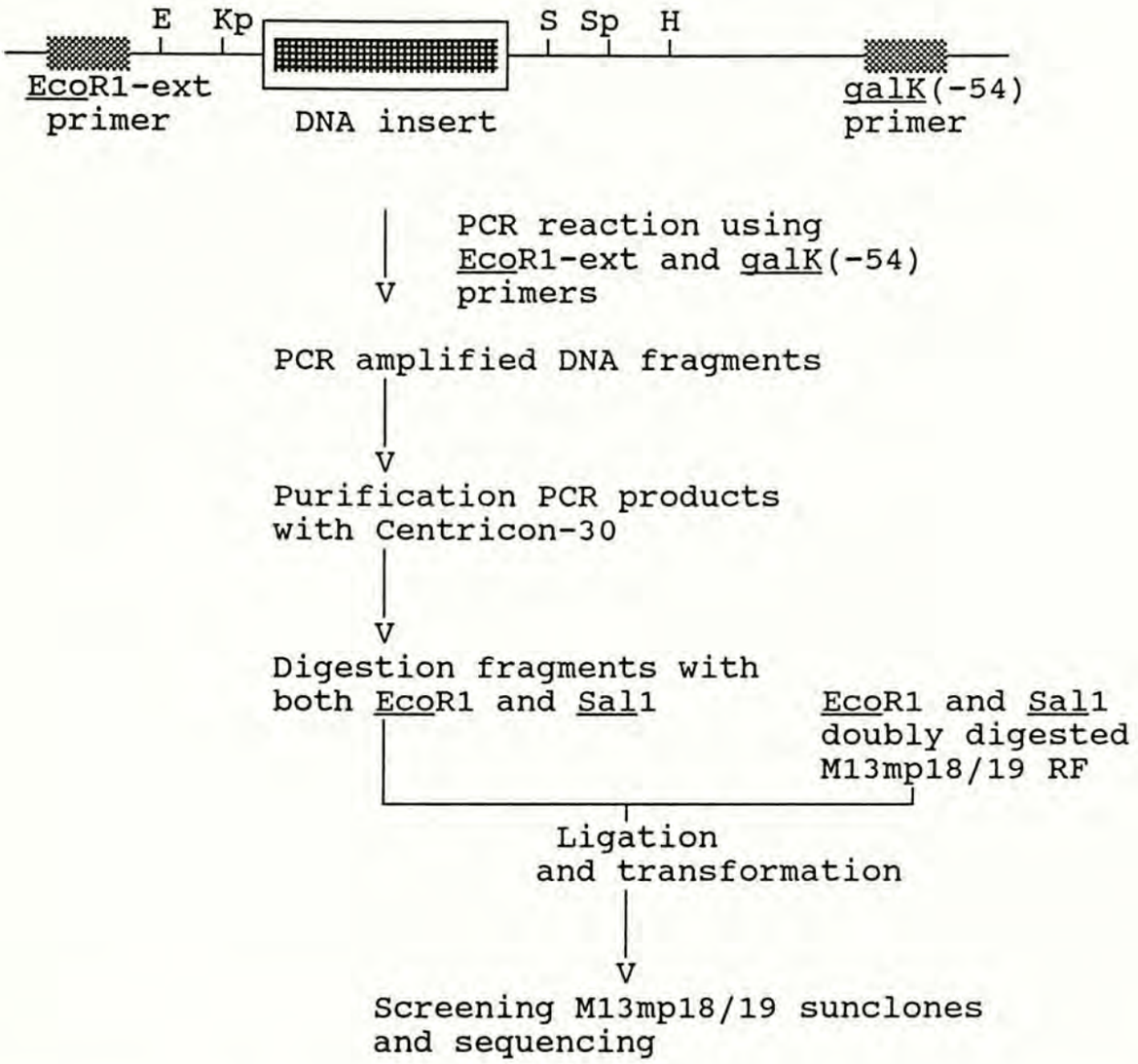


Figure 20. Subcloning and sequencing strategy of pFS1, pFS22 and pFS34.

```
          10          20          30          40          50
5' GATCTAACGC CTTTAACATC AAATTTCCGA GGTTATGACC AGAAAGTTCG
   Sau3A
          60          70          80          90         100
   CCATTACCGC CAAAACGATA CTCAAACATA GCGGACGACG CTAGGTTCGG

         110         120         130         140         150
   TAATTAACTG ATTCAGACAG TTGCGCATAT CGCCCCAGGT CCCGCCTTCC

         160         170         180         190         200
   GAGCGGCGAA TACGGCCAGT TGACCCCCCT TATCTGTGGT AGTCACAATG

         210         220         230         240         250
   CCGGTTCACG CGAACCTAAT GAAGAAAGCA GGAGAGGACG CTCTAATCCA

         260         270         280         290         300
   TGCCCTCCGC CGAGGGAACG ACACGATCAA GGTCAGCGTA CGATTGCGCA

         310         320         330         340         350
   TAAAAATTCC TGAAGTCAAA TAAGCAGCGC TACAGTAGCG CAAGTCGCGG

         360         370         380         390         400
   GTTTTCAGCA ATTAGCCGAC CGGCTAAAAG ACAATTAATT ATTCCCGACT

         410         420         430         440         450
   ACCCCTTTAT AGCGAGGGCG TTACCACGGC TAAAATGATA TATATCAATG

         460         470         480         490
   TAAAAGTGTG ATTTTCACGT ATTCTGTCGC TAAATAGCAC GATC 3'
                                                Sau3A
```

Figure 21. Nucleotide sequence of 494 bp Sau3A-Sau3A fragment in pFS22.

```
          10          20          30          40          50
5' GATCCTGTTC TTTGGATTCA TCACATTTAT TAGTTGGCTT ACGTAACTAG
   Sau3A
          60          70          80          90         100
   TTGGAATATT GCAAATTTGT GATGAAAGCT AGCATTTAGC TACGATGATT

         110         120         130         140         150
   TCATCAAACT GTTAACGTGC TACAATTGAA CTTGATATAT GTCAACGAAG

         160         170         180         190         200
   CGTAGTTTTA TTAGGTGTCC GGTACGTCTT AGCCTGTTAT GTTGCTGTTA

         210         220         230         240         250
   AAATGGTTAG GATGACAGCC GTTTTTGACA CTGTCGGGTC CAGAGGGAAA

         260         270         280         290         300
   GTACCCACGA CCAAGCTAAT GATGTTGTTG ACGTTGATGG AAAGTGCATC

         310         320         330         340         350
   AAGAACGCAA TTACGTACTT TAGTCATGTT ACGCCGATCA TGTTAATTTG

         360         370         380         390         400
   CGACATGCAT CAGGCAGGTC AGGGACTTTT GTACTTCCTG TTTCGATTTA

         410         420         430         440         450
   GTTGGCAATT TAGGTAGCAA ACATGCAGAC CCCGCACATT CTTATCGTTG

         460         470         480         490         500
   AAGACGAGTT GGTAACACGC AACACGTTGA AAAGTATTTT CGAAGCGGAA

         510         520         530         540
   GGCTATGATG TATTCGAAGC GACAGATGGC GCGGAAATGC ATCAGATC 3'
                                                  Sau3A
```

Figure 22. Nucleotide sequence of 548 bp Sau3A-Sau3A fragment in pFS34.

IV. Summary and Discussion.

A. Trimming down the size of DNA fragment to smaller fragment which still contained anaerobiosis promoter.

In the subcloning and sequencing study, I have investigated promoter clones (pHSK1, pHSK8, pFS1, pFS22, pFS34, pFE29 and pFE117) with anaerobic induction ratios greater than 6. pHSK1, pHSK8, pFS1, pFS22 and pFS34 were S. typhimurium promoter clones; pFE29 and pFE117 were E. coli promoter clones.

I have trimmed down the size of large DNA fragments which contained anaerobiosis-inducible promoters. The 7.1 kb fragment in pHSK1 was trimmed down to 1.3 kb in pHSK11, while 3.6 kb fragment in pHSK8 was trimmed down to 2.0 kb in pFSH8. The smaller fragments still contained the anaerobiosis-inducible promoters and were sequenced. Since the sizes of DNA insert in pFS1, pFS22 and pFS34 was only 0.6, 0.5 and 0.6 kb respectively, I did not attempt to trim the size of the fragments but determined their whole nucleotide sequences. pFE29 and pFE117 contained the same promoter of glpT. Only the 1.6 kb DNA fragment in pFE29 was trimmed down to 0.72kb in pFE33 which still had the lacZ gene under the control of glpT promoter. Moreover, another plasmid pFE32 was generated which had the lacZ gene under the control of glpA promoters.

B. Nucleotide sequencing

The Dideoxy sequencing method developed by Sanger et al. (1977) was used throughout the study. Generally,

subcloning into M13 phages to generate single-stranded template for sequencing was employed. In some cases, double-stranded recombinant pFZY1 plasmids were used as template for sequencing. The double-stranded template had to be denatured. When double stranded template was used, the sequencing pattern in the X-ray film was generally lighter than that using single-stranded templates. A higher primer concentration was preferred for double-stranded template sequencing. Besides the type of templates, the quality of the template also affected the sequencing pattern. For poor quality of template such as contamination of chromosomal DNA or RNA, the sequencing pattern was in a dark background or sometimes unreadable. Thus during the preparation of single-stranded M13 template, the time of cell grown should be about 5-6 hours so that the cell was not to old to have cell lysis occurred which would contaminate the phage supernatant. Another problem happened during the sequencing was "Band compressions" which masked the correct sequence in a particular region of the gel. This is due to the ability of G residues to form secondary structures that are not fully denatured during electrophoresis. These stable intra-strand structures appear to involve either Hoogsteen G-G (Mizusawa et al., 1986) or standard Watson-Crick G-C base-pairing. The strength of these particular base pairs can be reduced by modification of the guanine ring to eliminate specific grups which participate in hydrogen bond formation. The substitution of a carbon for a nitrogen atom

at position 7 (as in 7-deaza guanine), or removal of the exocylclic 3-amino group of guanine (as in inosine), will preclude G-G base pair formation and the strength of a G-C base pair is reduced to essentially that of an A-T pair. Hence, when "Band compression" appeared, the reaction was repeated using the Deaza ^{T7}Sequencing^{TM} Mixes, which included deoxy/dideoxynucleotide mixtures with 7-deaza dITP or 7-deaza dGTP substituted for dGTP. An example of the sequence pattern was given in Figure 23.

C. Subcloning and sequencing strategy

Several strategies were used in subclonig and sequencing. The straightforward strategy was first to determine restriction map of the clone. The smaller restriction fragments were generated for subcloning and sequencing. This strategy was applied to the sequencing of pHSK1 and pHSK8. The restriction map of pHSK1 was determined and a 1.3 kb HindIII-HindIII fragemnt from pHSK1 was subcloned as pHSK11. The restriction map of the 1.3 kb fragment in pHSK11 was further determined. Then two HincII-HincII fragment was generated for subcloning in M13mp phages for sequencing. To confirm that the 1.3 kb HindIII-HindIII fragment from in pHSK11 originated from pHSK1, 1.3 kb HindIII-HindIII from pHSK1 was also subcloned in M13 phages and sequenced from both ends (data not shown). It was confirmed that the 1.3 kb fragment in pHSK11 originated from pHSK1.

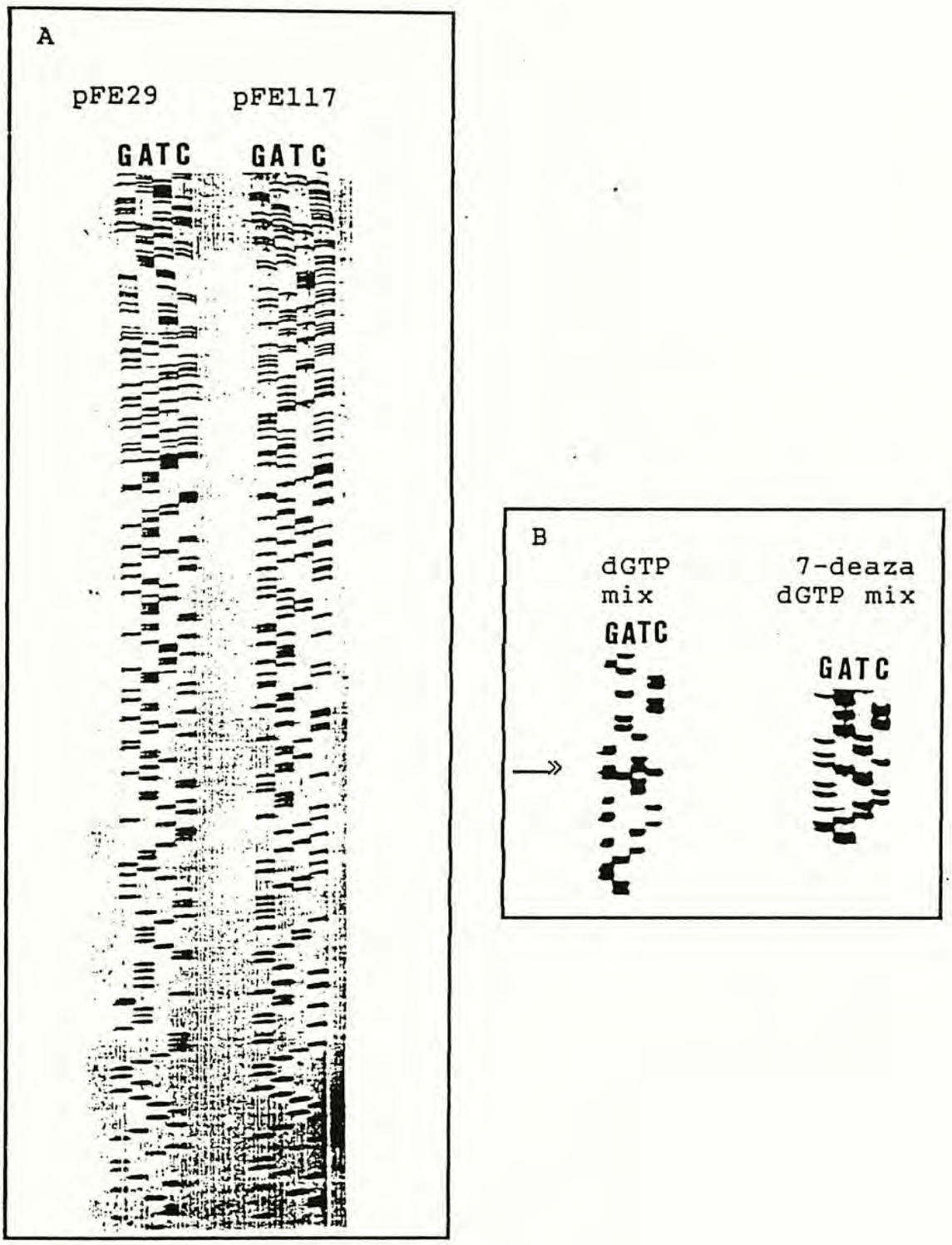


Figure 23. An example of sequence pattern and the use of 7-deaza dGTP nucleotide mix. A. Sequence pattern of pFE29 and pFE117 using M13mp single-stranded template. Dideoxynucleotide termination method was employed for sequencing reactions. Reaction products were analysed on 8% polyacrylamide gel. Direction of electrophoresis was from top to bottom. B. Use of 7-deaza dGTP nucleotide mix to solve band compression (indicated by ⟶»).

Another strategy was the generation of nested deletion by ExoIII unidirectional deletion, which was used during the sequencing of 2.0 kb EcoR1-Sal1 fragment in pHSK8. ExoIII nuclease only digests 5' overhangs but not 3' overhangs. Hence when the plasmid was digested by Sal1 and Pst1 (or other enzymes), a 5'overhang and a 3' overhang was generated respectively. ExoIII was then used to digest from the 5' overhang. To get a succesful result, the plasmid prepared for restriction enzyme digestion should mainly be supercoil-DNA. That is, without any nicks in the plasmid. Since the nicks in the plasmid would hold up some ExoIII, the extent of deletion by ExoIII would be difficult to follow.

The last strategy was the use of PCR to amplify fragments for subcloning and sequencing. This strategy was used for the subcloning and sequencing of pFS22 and pFS34 which contained small DNA inserts. Before doing the PCR reaction, two primers designed for PCR reaction was required. Thus the nucleotide sequence at both sides of the multi-cloning sites in pFZY1 was determined first. Double-stranded template of denatured pFZY1 was sequenced using pBR322 EcoR1 clockwise primer (5'-GTATCACGAGGCCCTT-3') and M13 forward (-40) primer (5'-GTTTTCCCAGTCACGAC-3'). The sequenced data was shown in Figure 24. From the nucleotide sequences, two primers was designed. One is called EcoR1-ext which is the pBR322 EcoR1 clockwise primer with 4 more bases. The other is called galK(-54) which is 54 bases from

Figure 24. Nucleotide sequence on both sides of the muticloning site in pFZY1. The unique cloning sites were marked. The site of the primers [EcoR1-ext and galK(-54)]designed for PCR reaction were underlined. The nucleotide sequence was determined with pBR322 EcoR1 clockwise primer and M13 forward (-40) primer.

the HindIII site. The sequence of galK(-54) primer did not have any homology greater than 50% with lacZYA or pBR322 as determined with the computer program DNASIS. The use of these two primer was successful to generate large amount of discrete DNA fragments from 100ng small scale prepared pFS22 and pFS34 plasmids. I have attempted to sequence the PCR products directly but failed. Perhaps, the PCR products we amplified were not clean enough for sequencing. As a result, the PCR products were subcloned into M13 phages for sequencing. During the subcloning, there was a problem in the subclones. The subclones sometimes contained concatamers of the inserts, especially when the subcloning fragments were of several species. This happened in the subcloning of HincII-HincII fragment from pHSK11 and in the subcloning of Sau3A-Sau3A fragments from 1.6 kb EcoR1-Sal1 fragment of pFE29. When pHSK11 was digested with HincII, four species of HincII-HincII fragments were generated. One of the M13 subclones was shown to be a concatamer of two HincII-HincII fragments when sequenced. On the other hand, when the purified 1.6 kb EcoR1-Sal1 fragment from pFE29 was degested with Sau3A, 9 species of Sau3A-Sau3A fragment was generated. One of subcloned pFE32 was shown to be a concatamer of 6 Species of the Sau3A-Sau3A fragments. I have dephosphorylated the Sau3A-Sau3A fragments with CIP before ligation. Hence, the dephosphorylation was not complete.

Chapter 4

Expression of anaerobiosis-inducible promoters

I. Introduction

The cloned promoters in pKK232.8 or pFZY1 were transcriptional fusions. The chloramphenicol acetyl transferase activity or ß-galactosidase activity reflected the transcription activities of the cloned promoters. In this chapter, the transcriptional expression of anaerobiosis-inducible promoters in pFE29, pFSH8, pFS22 and pFS34 were investigated.

Many anaerobiosis-inducible promoters are controlled by nitrate and/or the regulatory gene fnr(oxrA) (Stewart, 1988). The effects of nitrate and the regulatory gene fnr(oxrA) on the cloned anaerobiosis-inducible promoters were tested. During the isolation of anaerobiosis-inducible promoters, the E. coli strain MC1061-5 was used as host cells for transformation. So, the anaerobiosis-inducible properties of the promoters were in the genetic background of E. coli strain MC1061-5. In order to demonstrate the expression of S. typhimurium promoters cloned were also regulated by anaerobiosis in the genetic background of S. typhimurium strain, S. typhimurium strain JR501 was transformed by plasmids containing the anaerobiosis-inducible promoters of S. typhimurium.

Only one of these promoters was identified as

the promoter of *glpT* from *E. coli* by searching homology in the data base of Genbank (release 60.0) and EMBL (release 19.0). Thus, the unidentified promoters were likely to be some new cloned anaerobiosis-inducible promoters. By studying their expression, I hope to get some information about their function and speculate which gene the promoters were derived from.

II. Materials and methods

A. Bacterial strains and phages. The bacterial strains used in this study was listed in Table 16. P22-*int4* was used for the transduction in *S. typhimurium* and P1-*kc* was used for transduction in *E. coli*.

Table 16. Bacterial strains and plasmids

Strain	Genotype	Source or reference
Salmonella typhimurium LT2 derived strains		
HSK7	*oxrA*::Tn*10* *trp*::Tn*5*	Our lab strain
JR501	*hsdSA29* *hsdSB121* *hsdL6* *metA22* *metE551* *trpC2* *ilv452* H1-b H2-e *nix* *fla66* *nml* *rpsL120* *xyl404* *galE719*.	Tsai et al., 1989
HSK1508	As JR501 but with pFSH8	This study
HSK1522	As JR501 but with pFS22	This study
HSK1534	As JR501 but with pFS34	This study
HSK1608	As HSK1508 but *oxrA*::Tn*10*	This study
HSK1622	As HSK1522 but *oxrA*::Tn*10*	This study
HSK1634	As HSK1534 but *oxrA*::Tn*10*	This study
Escherichia coli K12 derived strains		
SK5022	*gal-25*, λ^-, *trpE63*, *zci-604*::Tn*10*, *fnr-1*, *rpsL195*, *icIR7*, *trpR72*	M. Deutscher strain
MC1061-5	*araD139*, Δ(*ara-leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *rpsL*, *hsr*	Koop et al., 1987
HSKE501	As MC1061-5 but *fnr*$^+$, *zci-604*::Tn*10*	This study
HSKE502	As MC1061-5 but *fnr-1*, *zci-604*::Tn*10*	This study
HSKE1500	As MC1061-5 but with pFE29	This study
HSKE1501	As HSKE501 but with pFE29	This study
HSKE1502	As HSKE502 but with pFE29	This study
HSKE1503	As MC1061-5but with pFE32	This study
HSKE1504	As MC1061-5 but with pFE33	This study

Table 16 cont'd

Plasmids[a]		
pFZY1	Ap^r, F'*lac* replicon, *lacZ*$^+$*Y*$^+$*A*$^+$	Koop et al., 1987
pFSH8	As pFZY1 but with a 2.0 kb DNA insert from *Salmonella typhimurium* LT2	This study
pFS22	As pFZY1 but with 0.49 kb DNA insert from *Salmonella typhimurium* LT2	This study
pFS34	As pFZY1 but with 0.54 kb DNA insert from *Salmonella typhimurium* LT2	This study
pFE29	As pFZY1 but with *glpT-lac* (1.6 kb DNA insert from *Escherichia coli*)	This study
pFE32	As pFZY1 but with *glpA-lac* (1.3 kb DNA insert from pFE29)	This study
pFE33	As pFZY1 but with *glpT-lac* (0.78 kb DNA insert from pFE29)	This study

a. The *lacZ*$^+$ genes of plasmid pFZY1 are actually *galK-lacZ* fusion but without promoter expressing the fusion as shown in Figure 2. Cell harboring plasmid pFZY1 appeared white in X-gal plate. Other plasmids are LacZ$^+$ with promoter expressing the *lacZ*$^+$ gene.

B. Media. NB consisted of 0.8% nutrient broth (Difco Laboratory). E medium consisted of 0.2 g $MgSO_4.7H_2O$, 2 g citric acid.$1H_2O$, 10 g K_2HPO_4 anhydrous, 3.5 g $Na(NH_4)HPO_4.4H_2O$, per liter (Vogel and Bonner, 1956). E medium was prepared as 50-fold concentrate and was diluted to final medium. Phage medium for p22 lysate preparation was made up of NB with 0.2% glucose and 1X E medium.

R medium consisted of 1% Bacto-tryptone, 0.1% Bacto-yeast extract and 0.8% NaCl. For R top agar, 0.6% Bacto-agar was added. For plate, 1.5% Bacto-agar was added. For P1 transduction, MC buffer used consisted of 0.1M $MgSO_4$, 0.005M $CaCl_2$ in distill water.

C. Transformation in S. typhimurium. Quick transformation in S. typhimurium LT2 was the method of Ryu and Martin (1990). S. typhimurium strain JR501 (Tsai et al., 1989) was used for the preparation of competent cells. Strain JR501 was grown to A_{510} of 0.2 (about $1X10^8$ cells/ml) in L-broth (Davis et al., 1980). The 1 ml culture was then transferred to a microcentrifuge tube and centrifuge for 1 min (15000rpm). The supernatant was removed and the cells were resuspended in 0.1ml ice-cold 100mM $CaCl_2$. The resuspended cells were kept on ice for 5 minute as competent cells for transformation. Plasmid DNA (10µl-100µl) was added to this competent cells, and immediately the competent cell with plasmid DNA added were heat-shocked for 1 min at 42°C. Part or all of the transformation mixture were plated

on L-agar whcih containing ampicllin (50µg/ml). The plates were incubated at 37°C overnight.

D. Genetic techniques

1. Preparation of P22 lysate of S. typhimurium strain HSK7 (oxrA::Tn10).

Method described by Davis et al. (1980) was employed to grow P22 lysate. Strain HSK7 was grown overnight in nutrient broth at 37°C with shaking. For 2 ml phage medium (NB + E medium and 1% glucose), 40µl overnight culture and 10µl phage (about $5x10^7$ pfu/ml) i.e. M.O.I. (multiplicity of infection) of about 0.01 was used. The mixture was grown overnight with shaking at 37°C. Then bacteria was lysed in tube by adding a few drops of chloroform and vortexing vigorously. Debris was precipitated with centrifuge and the supernatant (phage broth) was transferred to a screw cap tube with a few drops of chloroform added to the phage broth. The phage broth was kept in refrigerator for one or two hours before used.

2. Generalized transduction in S. typhimurium for the construction of oxrA::Tn10 containing strains.

The generalized transducing phage P22 int-4 used for generalized transduction. Transduction was performed according to the method of Ely et al. (1974). 0.1 ml of a freshly grown overnight culture of the recipient strain was pipetted onto the center of an appropriate transduction agar plate (with ampicillin to select the presence of plasmid

pKK232.8 or pFZY1 and tetracyline for the selection of oxrA::Tn10). Then 0.1 ml P22 lysate of strain HSK7 was added to the recipient culture. P22 lysate and bacterial culture were mixed by spreading with a bent glass rod. The plate was allowed to dry, and incubated at 37°C. Transductants were observed on the plate after 24 hours of growth.

3. Preparation of P1-kc lysates for generalized transduction in E. coli.

The method described by Miller (1972) was employed. Overnight culture was inoculated in 5ml LB broth containing 5 mM $CaCl_2$. The cell was grown at 37°C with shaking until the cell reached a density of $2x10^8$ cells/ml. To one ml culture, 10^7 P1-Kc phage was added and preadsorbed for 20 min in a 37°C. Then 3 ml R top agar (melted and kept at 45°C) was added and immediately plate onto a R plate. The plate was incubated at 37°C for 8 hours. At the end of this time, the soft agar layer was scrabed into a test tube and 5 drops of chloroform was added. The surface of the agar was washed with 1 ml broth and the broth was then added to the scrabed out top agar. The test tube was vortexed vigorously for 30 seconds and left at room temperature for 10 minutes. Then the agar and cell debris was centrifuge down, and the supernatant containing the P1 lysate was saved.

4. Transduction with P1 lysates.

One ml freshly grown overnight culture of the strain to be transduced was resuspended in 1 ml MC buffer. A

portion of 0.1 ml resuspended cells was transferred to each of three tubes. The first tubed was mixed with 0.1 ml P1 lysate. The second tube was mixed with a ten-fold diluted lysate. The third tbe was mixed with a hundred-fold diluted lysate. The mixture was left at 37°C for 20 min and then plated onto transduction plate.

III. Results

A. Expression of *glpT* promoter

The plasmid pFE29 contained the *glpT-lac* transcriptional fusion. ß-galactosidase activities of *E. coli* strain MC1061-5 harboring pFE29 were assayed from cell cultures grown in different media and culture conditions were assayed and shown in Table 17. The expression of *glpT* was induced for about six-fold under anaerobic condition. Anaerobiosis induction of *glpT* could be replaced by induction with the substrate glycerol-3-phosphate under aerobic condition. In addition, *glpT* was repressed by nitrate and glucose under anaerobic condition.

As discussed in Chapter 3, subcloning from *Sau*3A digested *Eco*R1-*Sal*1 fragment of pFE29 generated two subclones in pFZY1: pFE33 (containing the *glpT-lac* fusion) and pFE32 (containing the *glpA-lac* fusion). The expression of *glpT-lac* and *glpA-lac* in pFE32 were given in Table 18. The 0.78 kb fragment in pFE33 contained the necessary elements for anaerobiosis and glycerol-3-phosphate induction of *glpT*. However, the expression of *glpT* in pFE33 was no longer repressed by nitrate under anaerobic growth condition. The regulation of *glpA* expression in pFE32 was similar to that of *glpT*.

The effect of *fnr* mutation on *glpT* transcription was tested with isogenic strains (MC1061-5, *fnr*$^-$/*fnr*$^+$ *zci-604*::Tn*10*). The ß-galactosidase activity of *glpT-lacZ* fusion was decreased by *fnr* mutation (Table 19). The effect

of glucose and nitrate repression were the same for both strains. α-glycerol-3-phosphate still derepressed the expression of glpT under aerobic condition.

Table 17. Expression of Escherichia coli glpT promoter in pFE29 under various growth conditions.

Medium	ß-galactosidase sp act[b]		Induction ratio ($-O_2/+O_2$)
	Aerobic[a] ($+O_2$)	Anaerobic ($-O_2$)	
LBE	250	1200	4.8
LBE + 0.15 N NaCl	260	1300	5.0
LBE + 0.3 N NaCl	260	1200	4.6
LBE + 0.45 N NaCl	180	1100	6.0
LBE + G3P	1600	1600	1.0
LBE + Glucose	420	280	0.7
LBE + Nitrate	230	300	1.3
LBE + TMAO	231	1100	4.8

a. LBE is a buffered rich medium. The concentrations of the supplements were: G3P (α-glycerol-3-phosphate, 0.5%), Glucose (0.5%), Nitrate (0.5%) and TMAO (Trimethylamine-N-oxide, 0.5%).

b. The host strain was E. coli strain MC1061-5. ß-galactosidase specific activity was expressed as nmoles of ONP produced per minute per ml per A_{650}.

Table 18. Expression of *E. coli* *glpA-lac* in pFE32 and *glpT-lac* in pFE33.

Fusion[a]	Medium[b]	ß-galactosidase sp act[c]		Induction ratio ($-O_2/+O_2$)
		Aerobic ($+O_2$)	Anaerobic ($-O_2$)	
glpA-lac	LBE	54	580	11
	LBE + G3P	500	400	0.8
	LBE + Glucose	180	83	0.5
	LBE + Nitrate	50	440	9
glpT-lac	LBE	190	1200	6
	LBE + G3P	1900	1200	0.6
	LBE + Glucose	370	200	0.5
	LBE + Nitrate	480	1600	3

a. The host strain was MC1061-5.

b. LBE is a buffered rich medium. The concentrations of the supplements were: G3P (α-glycerol-3-phosphate, 0.5%), glucose(0.5%) and Nitrate(0.5%) .

c. ß-galactosidase specific activity was expressed as nmoles of ONP produced per minute per ml per A_{650}.

Table 19. Effect of *fnr* mutation on the expression of *glpT-lac* in pFE29.

Medium[a]	ß-galactosidase sp act[b]			
	Aerobic		Anaerobic	
	fnr$^+$	*fnr*$^-$	*fnr*$^+$	*fnr*$^-$
LBE	92	77	510	190
LBE + G3P	410	470	360	86
LBE + Glucose	7	44	11	18
LBE + Nitrate	65	44	150	140

a. LBE is a buffered rich medium. The concentration for the supplements were: G3P (α-glycerol-3-phosphate,0.5%), glucose(0.5%) and Nitrate(0.5%) .

b. The host strains were *fnr*$^+$ (MC1061-5, fnr$^+$, *zci-604*::Tn*10*) and *fnr*$^-$ (MC1061-5, *fnr*$^-$, *zci-604*::Tn*10*). ß-galactosidase sp act was expressed as nmoles of ONP produced per minute per ml per A_{650}.

B. Expression of S. typhimurium anaerobiosis-inducible promoters cloned in pHSK8, pFS22 and pFS34.

The expression of the anaerobiosis-inducible promoter cloned in pHSK8 was studied by subcloning a 2.0 kb fragments into pFZY1 plasmid as pFSH8. Plasmids pFSH8, pFS22 and pFS34 were transferred to S. typhimurium strain JR501 by transformation. The resulted strains were tested for the presence of the plasmids. oxrA::Tn10 mutation was then introduced into these strains via P22 transduction. The transductants were tested for oxrA phenotype by growing in nitrate medium anaerobically. The effect of nitrate and oxrA::Tn10 mutation on expression of the promoters in pFSH8, pFS22 and pFS34 in S. typhimurium strain JR501 was presented in Table 20.

The activity of the promoter in pFSH8 increased ten-fold when shifted from aerobic to anaerobic condition. The anaerobic activity was further increased three-fold by nitrate. Glucose had no significant effect on its expression.

The activity of the promoter in pFS22 was induced ten-fold by anaerobiosis, and the activity was further increased by six-fold in the presence of nitrate. On the other hand, the activity of the promoter in pFS34 was induced about ten-fold by anaerobiosis but unaffected by the presence of glucose or nitrate.

As regards the effect of oxrA::Tn10 mutation, nitrate induction of promoter in pFSH8 was eliminated by oxrA

mutation, while that of pFS22 was unaffected. Moreover, anaerobiosis-induction of promoter in pFS34 was also unaffected by oxrA mutation

Table 20. Effect of *oxrA* mutation on the expression of promoters in pFSH8, pFS22, and pFS34.

Plasmid	Medium[a]	ß-galactosidase sp act[c]			
		Aerobic[d]		Anaerobic	
		oxrA$^+$	*oxrA*$^-$	*oxrA*$^+$	*oxrA*$^-$
pFSH8[b]	LB	5	7	88	93
	LB + Glucose	5	5	80	79
	LB + Nitrate	4	5	449	120
pFS22	LB	3	9	64	68
	LB + Glucose	3	4	47	74
	LB + nitrate	3	11	471	625
pFS34	LB	34	40	293	259
	LB + Glucose	34	38	247	214
	LB + nitrate	35	31	258	216

a. The concentrations of the supplements were: glucose(0.5%) and Nitrate(0.5%) .

b. The host strain were JR501 (*oxrA*$^+$) and HSK1501 (JR501, *oxrA*::Tn*10*).

c. ß-galactosidase specific activity was expressed as nonomole ONP produced per min per ml per A_{650}.

d. Aerobic growth was achieved by inoculating 20µl overnight culture in LB into 1 ml test media in 16mm x 100mm test tube shaking vigorouly in rolling drum. Anaerobic growth was achieved by using anaerobic jar.

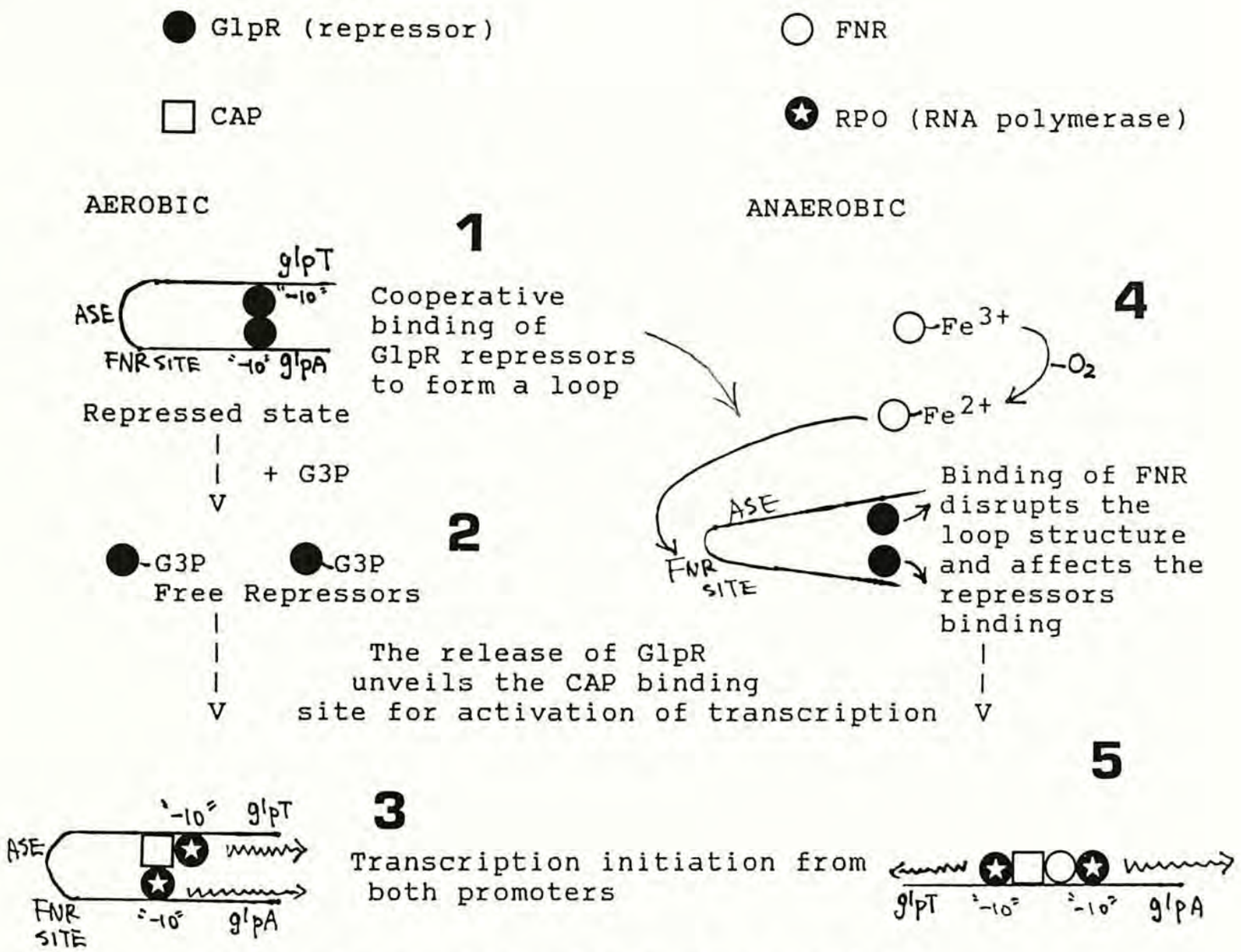
IV. Summary and Discussion

A. A pair of divergent promoters were both regulated by anaerobiosis and glucose.

Ehrmann et al. (1987) demonstrated that *glpABC* operon and *glpTQ* operon were transcribed divergently. The intergenic region between the start codons of the *glpT* and *glpA* genes consists of 272 bp (Eiglmeier et al., 1987). Kuritskes et al. (1984) isolated *glpA-lac* fusion and demonstrated that the transcriptional expression of *glpA* was induced by anaerobiosis. The anaerobic expression of *glpA* was decreased by *fnr* mutation. In this study, I have cloned the *glpT* promoter with *glpT-lac* fusion isolated. *glpT* was also regulated by anaerobiosis and the anaerobiosis induction was decreased by *fnr* mutation and nitrate. It seemed that a common regulatory element in the intergenic region between *glpA* and *glpT* may be responsible for the anaerobiosis induction for both *glpA* and *glpT* (Figure 25).

B. *fnr*(*oxrA*) dependent and independent promoters.

The effect of *fnr*(*oxrA*) on the expression of four cloned promoters were tested. Two of them were *fnr*-dependent and another two were *fnr*-independent. Previous studies have shown that there is at least two genetically distinct pathways for the regulation of anaerobiosis-inducible genes. One group is *fnr*-dependent and the other group is *fnr*-independent. For *fnr*-dependent genes, it is generally accepted that FNR act as a positive regulatory protein which



1. The intergenic region consists of ASE which facilitates the bending of the DNA. A loop is formed when GlpR repressors cooperatively bind to the operator sites. Transcription of both promoters are repressed.

2. Substrate a-glycerol-3-phosphate binds to the repressors and decreases the binding affinity of the repressors to the operators.

3. The release of the repressors from the operators allows the binding of CAP to the CAP binding site which overlaps with one of the operators. The binding of CAP then facilitates the binding of RNA polymerase to the promoters of both glpA and glpT to initiate transcription.

4. Under anaerobic condition, FNR-Fe^{3+} senses the redox state and changes to FNR-Fe^{2+} which then binds to the FNR binding site. The binding of FNR disrupts the loop structure. As a result, the repressors are released from the operators.

5. The binding of both FNR and CAP facilitate the binding of RNA polymerase to the promoters of both glpA and glpT to initiate transcription.

Figure 25. A proposed schematic model for the induction of divergent promoters ($glpA_p$ and $glpT_p$ of Escherichia coli)

binds to the promoter region to promote gene expression. For fnr-independent genes, ntrA (a sigma factor, Birkmann et al., 1987) or DNA supercoiling may be involved (Yamamoto and Droffner, 1985; Bhriain et al, 1989). Hence, the cloned promoters in this study should belong to two groups which are induced by anaerobiosis by two different mechanisms.

C. Effect of nitrate on anaerobiosis expression.

The expression of many anaerobiosis-inducible genes are affected by the presence of nitrate (Stewart, 1988). Some genes such as that coding for fumarate reductase are repressed by nitrate (Jones and Gunsalus, 1985), while some genes such as that coding for nitrate reductase are induced by nitrate (Fimmel and Haddock, 1979). The expression of glpT, when measured with the cloned 1.6kb fragment in pFE29 in this study, was repressed by nitrate. However, the expression of glpT when measured with a 0.72 kb fragment in pFE33 was no longer repressed by nitrate. comparing the 1.6 kb and 0.72 kb fragment, the 0.72 kb lost about 0.8 kb fragment which contained the coding region of glpA. Thus, this result indicated that part of the coding region of glpA may be responsible for the nitrate repression of glpT. Since narL is usually involved in nitrate regulation, a NarL binding site may be present in the coding region of glpA.

Two more promoters from S. typhimurium were also regulated by nitrate. They were induced by nitrate. These two cloned promoters were carried by pFSH8 and pFS22.

Interestingly, oxrA was required for the nitrate induction of the promoter carried by pFSH8 but was not required by that carried by pFS22. It seemed that nitrate induction was of two type - oxrA-dependent or oxrA-independent.

Stewart and Berg (1988) demonstrated that nitrate affects formate-hydrogen lyase synthesis by a pathway distinct from that for nitrate reductase and fumarate reductase. Fumarate reductase gene expression was insensitive to nitrate in narL insertion mutants. Nitrate only partially inhibited fumarate reductase sysnthesis in narX insertion mutants. On the other hand, nitrate fully inhibitted fumarate reductase synthesis in narK and narGHJI mutants. In contrast, narK and narGHJI insertion mutations significntly releved nitrate inhibition of formate-hydrogen lyase gene expression, while insertions in narL and narX had little impact on the effect of nitrate. The effect of nitrate on the expression of the promoters carried by pFSH8 and pFS22 may be analogous to that demonstrated by Stewart and Berg (1988).

Since the promoters in pFSH8 and pFS22 were induced by nitrate, they may both be involved in nitrate respiration. From previous studies, it was thought that enzyme synthesis of formate dehydrogenase-N (Lambden and Guest, 1976) was fnr-independent. However, Birkmann et al. (1987) showed that when the cell was grown under fully induced condition (anaerobically with nitrate), the enzyme synthesis of formate dehydrogenase-N was fnr-dependent. Thus, the regulation of the promoter carried by pFSH8 indicated that

this promoter may be the promoter of the gene coding for formate dehydrogenase-N. Another candidate gene from which the promoter carried by pFSH8 may be derived is narK, which is induced by nitrate. The expression of nark is dependent on the presence of fnr gene product (Stewart and Parales, 1989). The reason for this speculation is that part of the coding sequence downstream from the promoter carried by pFSH8 has homology with the DNA sequence of some DNA binding protein such as malT of E. coli. The gene product of narK was suggested to be a repressor (Stewart and Berg, 1988). However, further experiments are required to identify the origin of the promoters.

Since we do not have the strain with narL mutation, the effect of narL on nitrate regulation of the cloned promoters is not investigated.

Chapter 5

Analysis of anaerobiosis-inducible promoter containing DNA sequences and Final Discusssion.

I. Analysis of anaerobiosis-inducible promoter containing DNA sequences.

One of the major objectives of molecular biology has been to understand how gene expression is regulated. One way to study it is to analyze the rccognition sites bound by sequence-specific DNA-binding proteins, and to develop predictive correlations between nucleotide sequence homology and biological activity. With promoter probe plasmids pKK232.8 and pFZY1, I have sucessfully cloned and sequenced four anaerobiosis-inducible promoter containing DNA fragments from S. typhimurium, and one from E. coli as described in previous chapters.

In this chapter, I would analyse the sequences of the DNA fragments so as to identify any regulator elements in these anaerobisis-inducible promoters.

A. Search for initiation codons, conserved "-10" and "-35" regions.

Promoter sequence elements, determining the position of transcriptional initiation in E. coli, contain two regions of conserved DNA sequence located at about 10 and 35 nucleotides upstream from the transcription start site (the "-10" and "-35" sequences (Hawley and McClure, 1983; Harley and

Reynolds, 1987).

Since the DNA fragment contained in plasmid PFE29 is part of glpT sequence and glpA sequence, there is no need to identify the iniation codon and "-10" region because they have been identified by Cole and his colleague (Eiglmeier et al., 1987; Cole et al., 1988). glpT promoter does not have a conserved "-35" but a CAP site.

Using Hitachi's DNASIS computer program, the DNA sequences of all the sequenced fragments (1.3 kb in PHSK1, 1.98 kb in pHSK8, 494 bp in pFS22 and 548 bp in pFS34 were translated in all three reading frames. The results were given in Figures 26-29. In these Figures, the putative initiation codon, "-10" and "-35" were indicated. All the fragments contained an initiation codon except that of pFS22. All the fragments had a "-10" site. As regards "-35", only the fragment from pFS34 had a putative "-35".

For the 1329 bp insert in pHSK1, the third reading frame is likely to be the correct one and the codon ATG at 1324-1326 is the initiation codon. An evidence to support this was a translation fusion form between a HincII-HincII fragment from pHSK11 and lacZ gene in M13mp9. The recombinant M13 phage formed, in the absent of the lac inducer IPTG, pale blue plaques on X-gal plate ovelaid with a lawn of E. coli JM101 in soft agar. The blue color of the M13 plaque indicated that a translational fusion in frame with the lacZ gene was generated and under the control of that promoter of the inserted DNA fragment from pHSK1.

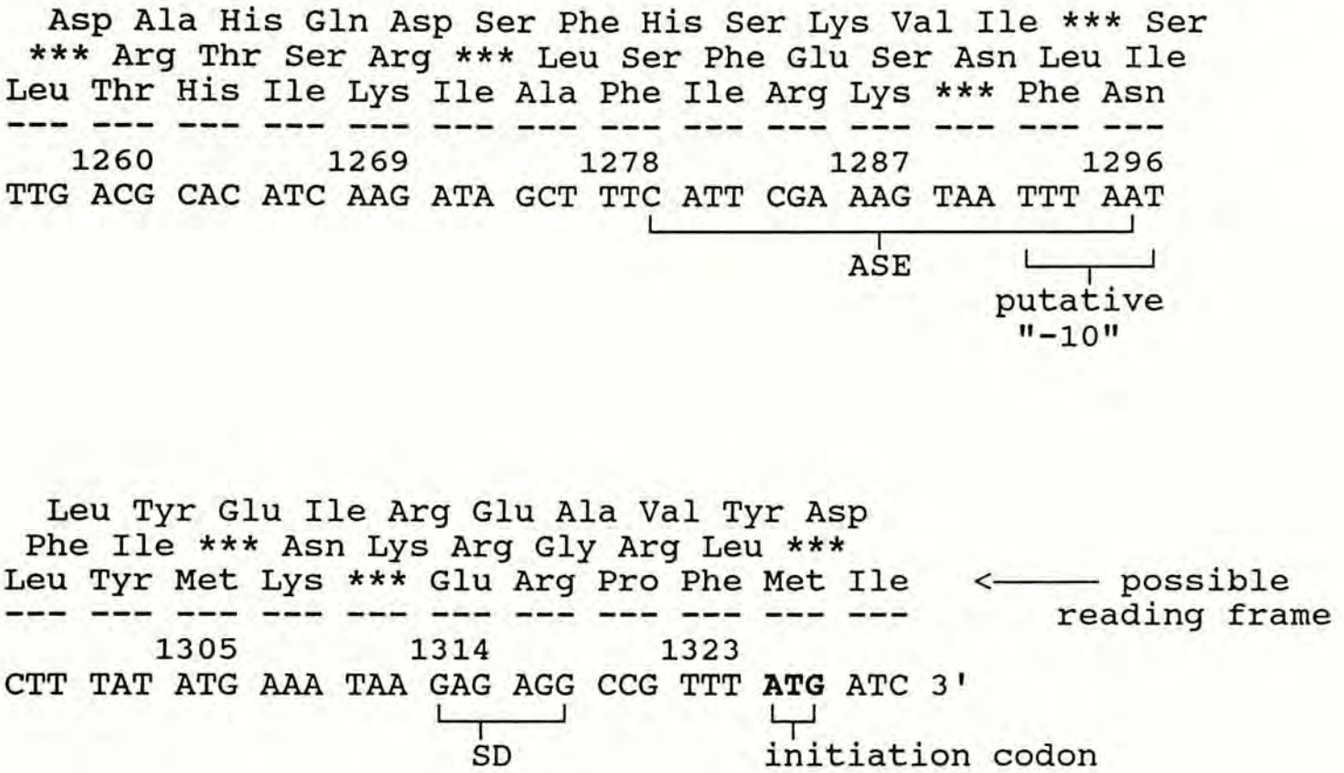


Figure 26. Nucleotide sequence of the 3' end (1255-1329) of the 1.3 kb HindIII-HindIII fragment of pHSK1 with DNA translation in three reading frame. The possible initiation codon, reading frame and regulatory elements are indicated. SD, Shinge Dalgarno sequence; ASE, anaerobic sense element suggested in this study. ***, stop codon.

```
  Gly Lys Ser Leu Glu Lys Arg Leu Gln *** Asn Leu Leu ***
 Arg Glu Val Val Gly Lys Glu Ile Ala Ile Lys Pro Val Ile
Ala Gly Ser Arg Trp Lys Arg Asp Cys Asn Lys Thr Cys Tyr
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
       1413        1422        1431        1440
GCG GGA AGT CGT TGG AAA AGA GAT TGC AAT AAA ACC TGT TAT
                  └──────────────┬──────────────┘
                          putative Fnr binding site

  Ala Ser Pro Ala Ser Val Asp Asn Leu Phe Leu Gln Ile Trp
 Gly Phe Pro Gly Leu Cys Gly *** Pro Val Leu Thr Asn Met
Arg Leu Pro Arg Pro Leu Trp Ile Thr Cys Ser Tyr Lys Tyr
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
 1450      1458        1467        1476        1485
AGG CTT CCC CGG CCT CTG TGG ATA ACC TGT TCT TAC AAA TAT
                        └──┬──┘
                         "-10"

  Ser Asp His Asp Thr Ala Cys Asp Glu Thr Arg Trp Leu ***
 Glu *** Ser *** His Arg Met *** *** Asn Glu Met Ala Val
Gly Val Ile Met Thr Pro His Val Met Lys Arg Asp Gly Cys
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
   1494        1503        1512        1521        1530
GGA GTG ATC ATG ACA CCG CAT GTG ATG AAA CGA GAT GGC TGT
└──┬──┘     └┬┘
  SD      initiation codon

  Ser Ala Ile Gln Ile Arg Ala His *** Gly Ser His Ser Thr
 Lys Cys His Ser Asn Gln Ser Ala Leu Arg Lys Pro Phe Tyr
Lys Val Pro Phe Lys Ser Glu Arg Ile Lys Glu Ala Ile Leu
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
       1539        1548        1557        1566
AAA GTG CCA TTC AAA TCA GAG CGC ATT AAG GAA GCC ATT CTA
└──────────┬──────────┘
          ASE

   Cys Thr *** Ser Ser Gly Ser Arg *** Arg Arg Leu Leu Cys
  Val His Val Lys Gln Arg Glu Ser Met Thr Gln Ile Thr Val
 Arg Ala Arg Lys Ala Ala Gly Val Asp Asp Ala Asp Tyr Cys
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1575        1584        1593        1602        1611
 CGT GCA CGT AAA GCA GCG GGA GTC GAT GAC GCA GAT TAC TGT

  Arg Arg Arg Ser Arg *** Gln Pro Asn Glu Arg Ala Gln Ser
 Pro Ser Gln Lys Ser Leu Ala Ala Lys *** Thr Arg Ala Val
Ala Val Ala Glu Val Val Ser Ser Gln Met Asn Ala Arg Ser
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
   1620        1629        1638        1647        1656
GCC GTC GCA GAA GTC GTT AGC AGC CAA ATG AAC GCG CGC AGT
```

Figure 27. pHSK8 cont'd

```
  Gly Gly Tyr *** Arg Asp Pro Asn Cys Gly *** Lys Pro Thr
 Arg Trp Ile Leu Thr Arg Ser Lys Leu Arg Leu Lys Thr Asn
Gln Val Asp Ile Asn Glu Ile Gln Thr Ala Val Glu Asn Gln
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
       1665        1674        1683        1692
CAG GTG GAT ATT AAC GAG ATC CAA ACT GCG GTT GAA AAC CAA


   Asp Val Arg Pro Val Gln Thr Ala Cys Pro Arg Leu His Arg
  *** Cys Pro Ala Arg Thr Asn Ser Leu Pro Ala Pro Thr Ser
 Leu Met Ser Gly Pro Tyr Lys Gln Leu Ala Arg Ala Tyr Ile
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1701        1710        1719        1728        1737
 CTG ATG TCC GGC CCG TAC AAA CAG CTT GCC CGC GCC TAC ATC


  Ile Pro Ser Arg Ser Arg His Ser Ala *** Lys Ala Trp Ser
 Asn Thr Val Thr Ile Ala Thr Phe Ser Val Lys Ser Val Val
Glu Tyr Arg His Asp Arg Asp Ile Gln Arg Glu Lys Arg Gly
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
   1746        1755        1764        1773        1782
GAA TAC CGT CAC GAT CGC GAC ATT CAG CGT GAA AAG CGT GGT


  Ser Glu Pro Gly Asn Ser Arg Pro Gly Arg Ala Asn *** Leu
 Val *** Thr Arg Lys Phe Ala Ala Trp *** Ser Lys Leu Thr
Arg Leu Asn Gln Glu Ile Arg Gly Leu Val Glu Gln Thr Asn
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
       1791        1800        1809        1818
CGT CTG AAC CAG GAA ATT CGC GGC CTG GTA GAG CAA ACT AAC


   Arg Val Ala Gln *** Lys Arg Gln Gln Arg Gln *** Ser His
  Pro Arg Cys Ser Met Lys Thr Pro Thr Lys Thr Val Lys Ser
 Ser Ala Leu Leu Asn Glu Asn Ala Asn Lys Asp Ser Lys Val
 --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1827        1836        1845        1854        1863
 TCC GCG TTG CTC AAT GAA AAC GCC AAC AAA GAC AGT AAA GTC


  Ser His Pro Ala Arg Phe Ala Gly Arg Asp Cys Arg Gln Thr
 Phe Pro Pro Ser Ala Ile Cys Trp Pro Gly Leu Ser Pro Asn
Ile Pro Thr Gln Arg Asp Leu Leu Ala Gly Ile Val Ala Lys
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
   1872        1881        1890        1899        1908
ATT CCC ACC CAG CGC GAT TTG CTG GCC GGG ATT GTC GCC AAA


  Leu Cys Pro Pro Ala Pro Val Ala Ala *** Arg Ser Thr His
 Thr Met Pro Ala Ser Thr Cys Cys Arg Val Thr *** Tyr Thr
His Tyr Ala Arg Gln His Leu Leu Pro Arg Asp Val Val His
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
       1917        1926        1935        1944
CAC TAT GCC CGC CAG CAC CTG TTG CCG CGT GAC GTA GTA CAC
```

Figure 27. pHSK8 cont'd

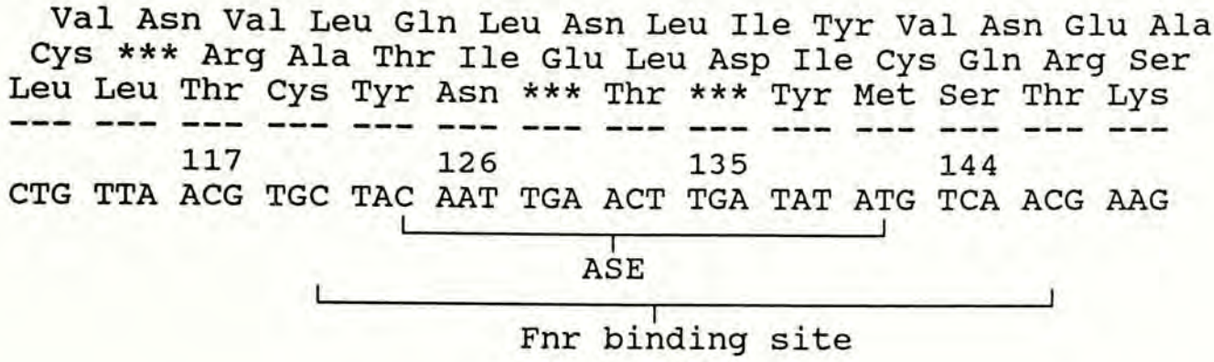
```
   Ala *** Ala Arg Arg Tyr Pro Leu Ser Arg
  Arg Met Ser Ala Ala Ile Ser Thr Ile Thr Ile
 Thr His Glu Arg Gly Asp Ile His Tyr His Asp   <----possible
 --- --- --- --- --- --- --- --- --- --- --- -     reading frame
1953        1962        1971        1980
 ACG CAT GAG CGC GGC GAT ATC CAC TAT CAC GAT C 3'
```

Figure 27. Nucleotide sequence of the 3' end (1405-1984) of the 2.0 kb EcoR1-Sal1 fragment of pHSK8 with DNA translation in three reading frames. The possible initiation codon, reading frame and regulatory elements are indicated. SD, Shinge Dalgarno sequence; ASE, anaerobic sense element suggested in this study. ***, stop codon.

```
  *** Lys Thr Ile Asn Tyr Ser Arg Leu Pro Leu Tyr Ser Glu
 Leu Lys Asp Asn *** Leu Phe Pro Thr Thr Pro Leu *** Arg
Ala Lys Arg Gln Leu Ile Ile Pro Asp Tyr Pro Phe Ile Ala
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
    378         387         396         405         414
GCT AAA AGA CAA TTA ATT ATT CCC GAC TAC CCC TTT ATA GCG
    └──────────────────────┘
            ASE

  Gly Val Thr Thr Ala Lys Met Ile Tyr Ile Asn Val Lys Val
 Gly Arg Tyr His Gly *** Asn Asp Ile Tyr Gln Cys Lys Ser
Arg Ala Leu Pro Arg Leu Lys *** Tyr Ile Ser Met *** Lys
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
        423         432         441         450
AGG GCG TTA CCA CGG CTA AAA TGA TAT ATA TCA ATG TAA AAG
          └──────────────────────────────────────┘
                        Fnr binding site

  *** Phe Ser Arg Ile Leu Ser Leu Asn Ser
 Val Ile Phe Thr Tyr Ser Val Ala Lys ***
Cys Asp Phe His Val Phe Cys Arg *** Ile
--- --- --- --- --- --- --- --- --- ---
459         468         477         486
TGT GAT TTT CAC GTA TTC TGT CGC TAA ATA
              └──┘
              "-10"

  Thr Ile
 His Asp
Ala Arg
--- --- --
       494
GCA CGA TC 3'
```

Figure 28. Nucleotide sequence of the 3' end (373-494) of the 0.49kb Sau3A-Sau3A fragment of pFS22 with DNA translation in three reading frame. No possible initiation codon and reading frame is identified. Putative regulatory elements are indicated. ***, stop codon.

Figure 29. pFS34



*** Phe Tyr *** Val Ser Gly Thr Ser *** Pro Val Met Leu
Val Val Leu Leu Gly Val Arg Tyr Val Leu Ala Cys Tyr Val
Arg Ser Phe Ile Arg Cys Pro Val Arg Leu Ser Leu Leu Cys
153 162 171 180 189
CGT AGT TTT ATT AGG TGT CCG GTA CGT CTT AGC CTG TTA TGT

Leu Leu Lys Trp Leu Gly *** Gln Pro Phe Leu Thr Leu Ser
Ala Val Lys Met Val Arg Met Thr Ala Val Phe Asp Thr Val
Cys Cys *** Asn Gly *** Asp Asp Ser Arg Phe *** His Cys
198 207 216 225 234
TGC TGT TAA AAT GGT TAG GAT GAC AGC CGT TTT TGA CAC TGT

Gly Pro Glu Gly Lys Tyr Pro Arg Pro Ser *** *** Cys Cys
Gly Ser Arg Gly Lys Val Pro Thr Thr Lys Leu Met Met Leu
Arg Val Gln Arg Glu Ser Thr His Asp Gln Ala Asn Asp Val
243 252 261 270
CGG GTC CAG AGG GAA AGT ACC CAC GAC CAA GCT AAT GAT GTT

*** Arg *** Trp Lys Val His Gln Glu Arg Asn Tyr Val Leu
Leu Thr Leu Met Glu Ser Ala Ser Arg Thr Gln Leu Arg Thr
Val Asp Val Asp Gly Lys Cys Ile Lys Asn Ala Ile Thr Tyr
279 288 297 306 315
GTT GAC GTT GAT GGA AAG TGC ATC AAG AAC GCA ATT ACG TAC

*** Ser Cys Tyr Ala Asp His Val Asn Leu Arg His Ala Ser
Leu Val Met Leu Arg Arg Ser Cys *** Phe Ala Thr Cys Ile
Phe Ser His Val Thr Pro Ile Met Leu Ile Cys Asp Met His
324 333 342 351 360
TTT AGT CAT GTT ACG CCG ATC ATG TTA ATT TGC GAC ATG CAT

Figure 29. pFS34 cont'd

```
  Gly Arg Ser Gly Thr Phe Val Leu Pro Val Ser Ile *** Leu
 Arg Gln Val Arg Asp Phe Cys Thr Ser Cys Phe Asp Leu Val
Gln Ala Gly Gln Gly Leu Leu Tyr Phe Leu Phe Arg Phe Ser
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
        369          378          387          396
CAG GCA GGT CAG GGA CTT TTG TAC TTC CTG TTT CGA TTT AGT


  Ala Ile *** Val Ala Asn Met Gln Thr Pro His Ile Leu Ile
 Gly Asn Leu Gly Ser Lys His Ala Asp Pro Ala His Ser Tyr
Trp Gln Phe Arg *** Gln Thr Cys Arg Pro Arg Thr Phe Leu
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
405          414          423          432          441
TGG CAA TTT AGG TAG CAA ACA TGC AGA CCC CGC ACA TTC TTA


  Val Glu Asp Glu Leu Val Thr Arg Asn Thr Leu Lys Ser Ile
 Arg *** Arg Arg Val Gly Asn Thr Gln His Val Glu Lys Tyr
Ser Leu Lys Thr Ser Trp *** His Ala Thr Arg *** Lys Val
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
    450          459          468          477          486
TCG TTG AAG ACG AGT TGG TAA CAC GCA ACA CGT TGA AAA GTA
                                           └──┬──┘
                                            "-35"


  Phe Glu Ala Glu Gly Tyr Asp Val Phe Glu Ala Thr Asp Gly
 Phe Arg Ser Gly Arg Leu *** Cys Ile Arg Ser Asp Arg Trp
Phe Ser Lys Arg Lys Ala Met Met Tyr Ser Lys Arg Gln Met
--- --- --- --- --- --- --- --- --- --- --- --- --- ---
        495          504          513          522
TTT TCG AAG CGG AAG GCT ATG ATG TAT TCG AAG CGA CAG ATG
                      └──┬──┘      └─┬─┘      └─┬─┘
                       "-10"         SD      initiation codon


  Ala Glu Met His Gln Ile
 Arg Gly Asn Ala Ser Asp
Ala Arg Lys Cys Ile Arg  <---------- possible reading frame
--- --- --- --- --- --- --
531          540          548
GCG CGG AAA TGC ATC AGA TC 3'
```

Figure 29. Nucleotide sequence of the 3' end (109-548) of the 0.55 kb Sau3A-Sau3A fragment of pFS34 with DNA translation in three reading frames. The possible initiation codon, reading frame and regulatory elements are indicated. SD, Shinge Dalgarno sequence; ASE, anaerobic sense element suggested in this study. ***, stop codon.

The putative Shine-Dalgarno sequence and the "-10" elements at 1312-1317 and 1291-1296 respectively were underlined in Figure 26.

For the 1984 bp insert in pHSK8, the third reading frame was likely to be the correct one. The evident was similar to that for pHSK1. A translational fusion was formed between the EcoR1-Sal1 fragment from pHSK8 and the lacZ gene of M13mp phage. The recombinant M13mp phage formed, in the absent of lac inducer IPTG, pale blue plaques on X-gal plate ovelaid with a lawn of E. coli JM101 in soft agar. The blue color of the M13 plaque indicated that a translational fusion in frame with the lacZ gene was generated and under the control of that promoter of the inserted DNA fragment from pHSK8. From the third reading frame, an open reading frame consisting of 162 amino acids was observed with the initiation codon ATG at nucleotide position 1498-1500. The putative Shine-Dalgarno sequence and the "-10" element at 1489-1492 and 1467-1472 respectively were underlined in Figure 27.

From the translated amino acid sequences in three reading frame for pFS22, there was no ATG initiation codon in the 3' end which was not interrupted by any stop codons. Thus, the regulatory elements were likely to begin upstream of the 3' end of the insert. Thus, the fragment contained in pFS22 did not contain any coding region of the cloned promoter. The putative "-10" element at 470-475 was underlined in Figure 28.

Finally, the translated amino acids sequence from pFS34

indicated that the first reading frame and third reading frame would be the possible reading frames because these two frame contained a ATG initiation codon which was not interrupted by any stop codon to the 3' end. However, the third reading frame was more likely the correct reading frame because putative Shine-Dalgarno and the conserved "-10" region can be located in front of the initiation codon at position 526-528. The position of the putative Shine-Dalgarno sequence and the conserved "-10" region were indicated on Figure 29. Moreover, a putative "-35" region was also located but was 21 base from that of "-10" region. This was unlike the usual spacer lengths be tween the "-10" and "-35" which is 16-18 base.

B. Search for FNR binding site and NarL binding sites.

1. FNR binding sites

Consensus sequences to which the FNR protein may bind have been proposed on the basis of sequence homology in the 5'-noncoding region of FNR-dependent genes (Jayaraman et al., 1987; Li and DeMoss, 1987; Spiro and Guest, 1987; Eiglmeier et al, 1989). Recent experimental evidence has supported the role of this motif in FNR-mediated regulation. When the inverted repeat 5'-TTGATNNNNATCAA-3' in the nar operon is disrupted by a deletion, a loss of anaerobic induction occurs (Li and DeMoss, 1988). Furthermore, when a 24-bp sequence from the nirB promoter, containing this

inverted repeat, is transplanted to the promoter region of galP1 and placed in a lacZ expression vector, the hybrid promoter stimulates the expression of lacZ under anaerobic conditions (Jayaraman et al., 1988). Eiglmeier et al. (1989) suggested the consensus sequences to be a 28 dyad symmetry 5'-GNNAAANTTGATNNNNATCAANTTTNNC-3'. The dyad symmetry was suggested to contain a conserved half-site AAA-TTGAT which can be subdivided into inner TTGAT motif and the outer AAA motif (Eiglmeier et al., 1989).

Since in my study, two promoters (promoter of glpT and the promoter contained by pHSK8) were shown to be fnr(oxrA)-dependent, they were searched for the suggested FNR binding site. For glpT , a putative FNR binding site with nucleotide sequence 5'-GTGAAACGTGATTTCATGCGTCATTTTGA-3' was located 75 nucleotides upstream of the transcriptional startpoint. Underlines in the nucleotides of the putative FNR binding site were homology region with the consensus FNR binding site. The site had about 75% homology with the consensus sequence. The sequence contained only a single half-site but with the outer AAA motif well maintained. This site was the same suggested for the FNR-binding site of glpA (Eiglmeier et al., 1989). However this site is only 27 nucleotides upstream of the transcriptional startpoint of glpA. If this site is really the FNR binding site responsible for the anaerobiosis induction of glpT and glpA, this site can then promote the anaerobiosis induction of a promoter with transcriptional startpoint which is 27 or 75 nucleotides downstream from the FNR binding site. This

finding led me to propose that the FNR binding site be an enhancer-like element which can promote anaerobiosis expression of a promoter independent of its orientation and exerts its effect from a long distance. An example of this kind of bacterial enhancer-like activator element (BELE) in E. coli has been reported by Garciarrubio and Covarrubias (1987). In their report, they demonstrated that E. coli glnA gene promoter glnAp2 is acitviated by an element which can act bidirectionally and at variable distances over the DNA. They also demonstrated that the BELE has specificity. An unidentified gene with a promoter 80p uptream from the BELE of glnA does not respond to the element. Since 80p (sigma-70) and glnA (sigma-60) use different sigma factors, they suggested that the selectivity of the BELE is dependent upon distinguishing the proper sigma factor. It was suggested that NR_1 (gene product of ntrC, acts as an activator of glnA gene expression) recognized the bacterial-enhancer-like sequence and then interact with sigma-60 (product of ntrA) to promoter expression to elicit its promoter selectivity. Would this be the case for that of anaerobiosis induction ? FNR is the activator, would the FNR binding site be a BELE ? Would a sigma factor be required (would it be the sigma-32, sigma-60, sigma-70, or an unidentified one) ?. Further analysis is required to test this speculation.

In the nucleotide sequence from pHSK8, a putative FNR binding site was also located. The binding site had a

sequence of 5'-TGGAAAAGAGATTGCAATAAAACCTGTT-3. This site was located 53 nucleotides from the suggested initiation codon. Underlines in the nucleotides of the putative FNR binding sequence were homology region with the consensus FNR binding site. This site also had a 75% similarity to that of suggested FNR-binding site. . By the computer search, another putative FNR binding site was located at 247 nucleotides from the EcoR1 site of the sequenced EcoR1-Sal1 fragment. The site was 5'-CGAACTCTCGATGACCTTCAATTTTCAT-3'. This site had a 79% homology with the putative FNR consensus binding site. This site is located at 1.2 kb from the suggested initiation codon. If this site is functional, it would be an enhancer-like element. However, whether these two putative FNR binding sites are both functional or not has to await further analysis.

2. NarL binding site

In this study, the glpT promoter (p.131) and the promoters carried by pFSH8 and pFS22 (p.136) are regulated by nitrate. The nucleotide sequence of the DNA fragment carrying these promoters were searched for any NarL binding site.

When the 1.6 kb insert of pFE29 (the glpT promoter-containing fragment) was trimmed down as 0.78 kb as pFE33, the expression of glpT was no longer repressed by nitrate. Thus, the nucleotide sequence of the removed 0.9 kb fragment from pFE29 was searched for the presence of NarL binding sites. Based on proposed consensus NarL binding site

(Eiglmeier et al., 1989), three putative NarL binding sites were located and shown as follows:

```
   -438                        -414
1. 5'-GCAATGCGTTTCAGGATCTGGTTTT-3'

   -528                        -504
2. 5'-CGAAATGGATCAACGGTGCCATCCG-3'

   -546                        -522
3. 5'-ATGTTTGCTGCGGTCAGACGAAATG-3'
```

The numbers indicated above the sequences were the base pairs upstream of the transcription startpoint. However, these putative sites had poor homology (only about 50%) with the suggested consensus nucleotide sequence of NarL binding site. Since the effect of narL mutation has not been tested, the significance of these putative NarL binding sites is not certain. Moreover, nitrate repression as suggested by Stewart and Berg (1988) can be of two types — one involves narL and narX and the other one involves narK and narGHJI.

As regards to the promoters carried by pHSK8 and pFS22, these two promoters were induced by the presence of nitrate. Since narGHJI was also induced by nitrate and the sequence in the promoter responsible for nitrate induction by narL was located (Li and DeMoss, 1988), the located sequence 5'-AATATGTTACCCATGGGGAATACTC-3' was used for homology search in the nucleotide sequence of the fragment carried by pHSK8 and pFS22. The homology regions were as follows:

pHSK8

5'-AATATGTTGTATTAATTGACTACAA-3' which is located at 154 to 178 bp from the suggested initiation codon (Figure 26). This

region form a dyad symmetry with downstream nucleotide. The dyay symmetry is indicated by opposite arrows in the following sequence which includes some downstream sequence:

5'-AATATGTTGTATTAATTGACTACAATTGCTACAACACCTG-3'

pFS22

5'-AGCAATTAGCCGACCGGCTAAAAGA-3'

The located NarL bindig site in pFS22 by homology search also has a dyad symmetry. This findings suggested that secondary structure in the promoter sequences is important for regulation.

C. Homology search among the promoter sequences of all anaerobiosis-inducible genes.

The upstream regulatory sequences (Sawers and Bck, 1989) of pfl was compared with that of glpT since both of them are strongly expressed anaerobically and with high anaerobic induction ratios (6-10). A homology region was indentied as follows:

glpT 5'-AATTCACATTTAATTTAT-3'

pfl 5'-AATTTGTATTTAATAAAT-3'

Then a motif of 18 bp 5'-AATTNNNATTTAATNNAT-3' was used for homology search among the 5' noncoding DNA sequence

of 14 known anaerobiosis-inducible genes (Figure 30) and the four unidentified promoters in this study. All the promoters had homology with this motif and the homology regions were aligned as shown in Figure 31. The suggested consensus sequence is called "anaerobiosis sense element" (ASE). It is suggested that homology among the controlled regions are likely to have a function (Studnicka, 1987), the ASE is likely to have a function. Further analysis is required to define the functional significancy of the suggested ASE on anaerobiosis regulation.

ack (Matsuyaam et al., 1989)

```
          10         20         30         40         50
5' ACGCGTCATCTTGATAACGCGATTTTCGACAAAGACCGGGGCAAGGCGTT
          60         70         80         90        100
   TTTCCAGCGGCCACGTCTTTGAGTAATGCTGTCCCCGGCGAAACAAGCTA
         110        120        130        140        150
   AAAAAATTAACAGAACGATTATCCGGCGTTGACATGCTTCACCTCAACTT
         160        170        180        190        200
   CACATATAAAGATTCAAAATTTGTGCAAATTCACAACTCAGCGGGACAAC
         210        220        230        240        250
   GTTCAAAACATTTTGTCTTCCATACCCACTATCAGGTATCCTTTAGCAGC
         260        270        280        290        300
   CTGAAGGCCTAAGTAGTACATATTCATTGAGTCGTCAAATTCATATACAT
         310        320        330        340        350
   TATGCCATTGGCTGAAAATTACGCAAAATGGCATAGACTCAAGATATTTC
         360        370        380        390        400
   TTCCATCATGCAAAAAAAATTTGCAGTGCATGATGTTAATCATAAATGTC
         410        420        430        440        450
   GGTGTCATCATGCGCTACGCTCTATGGCTCCCTGACGTTTTTTTAGCCAC
         460        470
   GTATCAATTATAGGTACTTCCATG    3'
```

ansB (Jennings and Beacham, 1990)

```
          10         20         30         40         50
5' GGTCGGGAATTTAAAATAATCCTCTATTTTAAGACGGCATAATACTTTTT
          60         70         80         90        100
   TATGCCGTTTAATTCTTCGTTTTGTTACCTGCCTCTAACTTTGTAGATCT
         110        120        130        140        150
   CCAAAATATATTCACGTTGTAAATTGTTTAACGTCAAATTTCCCATACAG
         160        170        180        190
   AGCTAAGGGATAATGCGTAGCGTTCACGTAACTGGAGGAATGAAATG 3'
```

aspA (Jerlstrom et al., 1987)

```
          10         20         30         40         50
5' GGCTACCTGAATGGGTTGCGAATCGCGTTTAGCTTATATTGTGGTCATTA
          60         70         80         90        100
   GCAAAATTTCAAGATGTTTGCGCAACTATTTTTGGTAGTAATCCCAAAGC
         110        120        130        140        150
   GGTGATCTATTTCACAAATTAATAATTAAGGGGTAAAAACCGACACTTAA
         160        170        180        190        200
   AGTGATCCAGATTACGGTAGAAATCCTCAAGCAGCATATGATCTCGGGTA
         210        220        230        240        250
   TTCGGTCGATGCAGGGGATAATCGTCGGTCGAAAAACATTCGAAACCACA
         260        270        280        290        300
   TATATTCTGTGTGTTTAAAGCAAATCATTGGCAGCTTGAAAAAGAAGGTT

   CACATG 3'
```

dmsA (Bilous et al., 1988)

```
          10        20        30        40        50
5' TCTCCCTTTGATACCGAACAATAATTACTCCTCACTTACACGTAATACTA
          60        70        80        90        100
   CTTTCGAGTGAAAATCTACCTATCTCTTTGATTTTCAAATTATTCGATGT
          110       120       130       140       150
   ATACAAGCCTATATAGCGAACTGCTATAGAAATAATTACACAATACGGTT
          160       170       180       190       200
   TGTTACTGGAATCAATCGTGAGGAAGCTTGAGTGAGCCATTATGAAAACG
          210       220       230       240       250
   AAAATCCCTGATGCGGTATTGGCTGCTGAGGTGAGTCGCCGTGGTTTGGT
          260       270       280
   AAAAACGACAGCGATCGGCGGCCTGGCAATG 3'
```

fdhF (Birkmann et al., 1987)

```
          10        20        30        40        50
5' CCGCTTACAGGCCACCGAAGCCCGGTTGCCAGTGCTTGCCGAAGAGATGT
          60        70        80        90        100
   CATTACTGATGCTGGACAGCCGCCGGGTGATCCAAAGCATTCAGTTGATG
          110       120       130       140       150
   AAATCGCTGGGCGGCGGGTATCAGGCAGGTCCCGTCGTCGAGAAAAAATA
          160       170       180       190       200
   AAATGTCTGCCGCGTGATGGCTGTCACGCGGTATTTCGTTTCGTCACGTC
          210       220       230       240       250
   AAAACTGACGACAGCCTGTTTTTCGTCAGAGTTTTGAATAAATAGTGCCC
          260       270       280       290       300
   GTAATATCAGGGAATGACCCCACATAAAATGTGGCATAAAAGATGCATAC
          310       320       330       340       350
   TGTAGTCGAGAGCGCGTATGCGTGATTTGATTAACTGGAGCGAGACCGAT

   G 3'
```

frdA (Cole, 1982)

```
          10        20        30        40        50
5' GCGTTTTAATAAGTTAGGAATGGATGCGCTCGGCTGCCAGGATGCCGTTT
          60        70        80        90        100
   CGCTCATAGTTAAATCTCCAGTTTTTGACAAGGGCACGAAGTCTACTCGC
          110       120       130       140       150
   AACGCGACGGCGAGACAAATTTTACGCAGGAATCAAACAGCGGTTGGCAG
          160       170       180       190       200
   TGACTAAAAAAAAGCACGATCTGATGGTTTAGTAATTAAATTAATCATCTT
          210       220       230       240       250
   CGATGATAATTTAGCCCTCTTGCGCACTAAAAAAATCGATCTCGTCAAAT
          260       270       280
   TTCAGACTTATCCATCAGACTATACTGTTGTACCTATA
```

fumB (Bell et al., 1989b)

```
          10         20         30         40         50
5' TTCCGGTCGCGCTGGCGATCGGCGTTGATCCGGCATACATCGTGGCTTCA
          60         70         80         90         100
   GCACCGGCTTGCTACGGTTATTACATCCTGCCGACTTATCCCAGCGATCT
         110        120        130        140        150
   GGCAGCGATTCAGTTTGACCGTTCCGGCACCACCCACATCGGTCGCTTCG
         160        170        180        190        200
   TCATCAACCACAGCTTTATTCTGCCGGGGTTGATTGGTGTGAGCGTATCG
         210        220        230        240        250
   TGCGTCTTCGGCTGGATCTTCGCCGCGATGTACGGGTTCTTATAAATGCA
         260        270        280        290        300
   CTTTGCGTGCCGCCCGTGACTACGCGGCACGCCATTTTCGAATAACAAAT
         310        320
   ACAGAGTTACAGGCTGGAAGCTATG 3'
```

gap (Branlant and Branlant, 1985)

```
          10         20         30         40         50
5' CAAAATTTTGTCCTAAACTTGATCTCGACGAAATGGCTGCACCTAAATCG
          60         70         80         90         100
   TGATGAAAATCACATTTTTATCGTAATTGCCCTTTAAAATTCGGGGCGCC
         110        120        130        140        150
   GACCCCATGTGGTCTCAAGCCCAAAGGAAGAGTGAGGCGAGTCAGTCGCG
         160        170        180        190        200
   TAATGCTTAGGCACAGGATTGATTTGTCGCAATGATTGACACGATTCGCT
         210        220        230        240        250
   TGACGCTGCGTAAGGTTTTTGTAATTTTACAGGCAACCTTTTATTCACTA
         260        270
   ACAAATAGCTGGTGGAATATATG 3'
```

glpA (Cole et al., 1988)

```
          10         20         30         40         50
5' GATCCCCGAAAGGGCAAAACCTAAATCACCGCGTGAGAATCCCTGCTCAA
          60         70         80         90         100
   CCAGATAAGGCATAGCAAGCGCAAAGTTCTTACGAACCAAATAGTAAGCC
         110        120        130        140        150
   GCATAGCCAAAGAATATCCCCAGGAAAATTTGCCAGCGCAATCGACGATA
         160        170        180        190        200
   AGTCGGATCGATCTCCGCGGCAGGTAAGCGCGCTTTGTGTGGCGCTGGTT
         210        220        230        240        250
   TAAAAATACTCAACATTGATAGCCTCCGTGGCCCGTGGTCTTATTTATGA
         260        270        280        290        300
   TTAACAGCCTGATTCAGTGAGAGAACCTGCCGTTTCTTGAGTTGCCGCGA
         310        320        330        340        350
   TGTTAAGAAAACATTCATAAATTAAATGTGAATTGCCGCACACATTATTA
         360        370        380        390        400
   AATAAGATTTACAAAATGTTCAAAATGACGCATGAATCACGTTTCACTTT
         410        420        430        440        450
   CGAATTATGAGCGAATATGCGCGAAATCAAACAATTCATGTTTTTACTAT
         460        470        480        490
   GGCTAAATGGTAAAAAACGAACTTCAGAGGGATAACAATG
```

narG (Li and DeMoss, 1988)

```
          10        20        30        40        50
5'      CCTGCGTAGTGATTACCTGGGCGGTATATGGTCGGCATTCTAAA
          60        70        80        90        100
AAATAAACCGTTACTCGTCATACTTCGGGTTACATGTGCTGCGGCTGCGT
          110       120       130       140       150
TCATTCACCCCAGTCACTTACTTTAGTAAGCTCCTGGGATTCATTCACTT
          160       170       180       190       200
GCCGCCTTCCTGTAAACCGAATTATATAGAGTAAAATATTTGATTATCCT
          210       220       230       240       250
TTGCGCGGCATGATGTCGCGCTTTTTTTATGCGTCATTTAGTTACAACAT
          260       270       280       290       300
ACTAATGTTATATGGTTTATTTCGCCGGATTTCATTAAGAGCCATTAATA
          310       320       330       340       350
TGTTACCCATGGGGAATACTCCTTAATACCCATCTGCATAAAAATCTTAA
          360       370       380       390       400
TAGTTTAAATAACTACAGGTATAAAACGTCTTAATTTACAGTCTGTTATG
          410       420       430       440       450
TGGTGGCTGTTAATTATCCTAAAGGGGTATCTTAGGAATTTACTTTATTT
          460       470       480       490       500
TTCATCCCCATCACTCTTGATCGTTATCAATTCCCACGCTGTTTCAGAGC
          510       520
GTTACCTTGCCCTTAAACATTA
```

nirB (Jayaraman et al., 1987)

```
          10        20        30        40        50
5' CGGCCTTTTCCTCTCTTCCCCCGCTACGTGCATCTATTTCTATAAACCCG
          60        70        80        90        100
CTCATTTTGTCTATTTTTTGCACAAACATGAAATATCAGACAATTCCGTG
          110       120       130       140       150
ACTTAAGAAAATTTATACAAATCAGCAATATACCCATTAAGGAGTATATA
          160       170       180       190       200
AAGGTGAATTTGATTTACATCAATAAGCGGGGTTGCTGAATCGTTAAGGT
          210       220       230
AGGCGGTAATAGAAAAGAAATCGAGGCAAAAATG 3'
```

pepN (Bally et al., 1986)

```
            10         20         30         40         50
5' ACGGCTTGCTGCATATGCAACTTATAAGCATCTGTATCCAGCAACGAGTG
            60         70         80         90        100
   CAGAACAGGAGAAGCGAATTGTGTCATAGGTGCGCAGTAGCGTCCTCGTA
           110        120        130        140        150
   CAGGAGCGTTTAGTACAATAAACATCTTCAGGAACTGCTGGAGTATACCT
           160        170        180        190        200
   TGTTTCGCAATTTATTGAACCCCGATCACACCATATGCCACCTTTCTTGG
           210        220        230        240        250
   TCGAGGCATTACGCGGTGTATGTTATAAAAATGTAGCAATAAAGGCGTTT
           260        270        280        290        300
   GTACCTGAAAAGATGAAGATTCTGCATAGCGCGATTTACGCAACAGGAAT
           310        320        330        340
   AGACTGAACACCAGACTCTATAAAAGATGCTAAAGGTTATTTATG 3'
```

pfl (Sawers and Böck, 1988):

```
            10         20         30         40         50
5' TTGCTGCACATCAGTCGTTGTTGAAGGCCTACGAAAAGCTGCAGCGCGCC
            60         70         80         90        100
   AAAGCAGCATTCTGGGCAAAATAAAATCAAATAGCCTACGCAATGTAGGC
           110        120        130        140        150
   TTAATGATTAGTCTGAGTTATATTACGGGGCGTTTTTTTAATGCCCCGCT
           160        170        180        190        200
   TTACATATATTTGCATTAATAAAATAATTGTAATTATAAGGTTAAATATC
           210        220        230        240        250
   GGTAATTTGTATTTAATAAATACGATCGATATTGTTACTTTATTCGCCTG
           260        270        280        290        300
   ATGCTCCCTTTTAATTAACTGTTTTAGCGGAGGATGCGGAAAAAATTCAA
           310        320        330        340        350
   CTCATTTGTTAATTTTTAAAATTTATTTTTATTTGGATAATCAAATATTT
           360        370        380        390        400
   ACTCCGTATTTGCATAAAAACCATGCGAGTTACGGGCCTATAAGCCAGGC
           410        420        430        440        450
   GAGATATGATCTATATCAATTTCTCATCTATAATGCTTTGTTAGTATCTC
           460        470        480        490        500
   GTCGCCGACTTAATAAAGAGAGAGTTAGTGTGAAAGCTGACAACCCTTTT
           510        520        530        540        550
   GATCTTTTACTTCCTGCTGCAATGGCCAAAGTGGCCGAAGAGGCGGGTGT
           560        570        580        590        600
   CTATAAAGCAACGAAACATCCGCTTAAGACTTTCTATCTGGCGATTACCG
           610        620        630        640        650
   CCGGTGTTTTCATCTCAATCGCATTCGTCTTCTATATCACAGCAACCACT
           660
   GGCACAGGCACAATG 3'
```

Figure 30. Nucleotide sequence of the 5' noncoding region of 13 known anaerobiosis-inducible genes. The initiation codon for each gene is included at the 3' end of the given sequence, except frdA and narG where the 3' ends are the transcriptional startpoints.

ackA	A	C	T	T	C	A	C	A	T	A	T	A	A	A	G	A	T	T	164
ansB	A	A	T	A	A	T	C	C	T	C	T	A	T	T	T	T	A	A	32
aspA	A	A	T	T	A	A	T	A	A	T	T	A	A	G	G	G	G	T	134
dmsA-1	A	T	T	T	T	C	A	A	A	T	T	A	T	T	C	G	A	T	98
dmsA-2	A	A	C	T	G	C	T	A	T	A	G	A	A	A	T	A	A	T	136
fdhF	A	G	T	T	T	T	G	A	A	T	A	A	A	T	A	G	T	G	246
frdA-1	T	T	T	A	G	T	A	A	T	T	A	A	A	T	T	A	A	T	194
frdA-2	A	A	T	T	T	C	A	G	A	C	T	T	A	T	C	C	A	T	265
fumB	T	T	A	T	A	A	A	T	G	C	A	C	T	T	T	G	C	G	256
gap	A	A	T	T	G	C	C	C	T	T	T	A	A	A	A	T	T	C	92
glpA-1	A	A	A	T	A	A	G	A	T	T	T	A	C	A	A	A	A	T	367
glpA-2	A	A	T	T	C	A	T	G	T	T	T	T	T	A	C	T	A	T	450
glpT-1	A	A	T	T	C	A	C	A	T	T	T	A	A	T	T	T	A	T	400
glpT-2	A	A	A	T	C	T	T	A	T	T	T	A	A	T	A	A	T	G	383
glpT-3	A	A	A	C	G	T	G	A	T	T	T	C	A	T	G	C	G	T	348
narG-1	C	A	T	T	A	A	T	A	T	G	T	T	A	C	C	C	A	T	300
narG-2	C	T	T	A	G	G	A	A	T	T	T	A	C	T	T	T	A	T	448
nirB-1	A	A	T	T	T	G	A	T	T	T	A	C	A	T	C	A	A	T	170
nirB-2	A	A	G	G	T	G	A	A	T	T	T	G	A	T	T	T	A	C	168
pepN	T	T	T	C	G	C	A	A	T	T	T	A	T	T	G	A	A	C	170
pfl-1	A	A	T	T	T	G	T	A	T	T	T	A	A	T	A	A	A	T	221
pfl-2	A	A	T	T	T	A	T	T	T	T	T	A	T	T	T	G	G	A	337
pfl-3	T	A	T	T	T	G	C	A	T	A	A	A	A	A	C	C	A	T	370
pHSK1	G	A	T	T	C	G	A	A	A	G	T	A	A	T	T	T	A	A	1295
pHSK8	A	A	G	T	G	C	C	A	T	T	C	A	A	A	T	C	A	G	1549
pFS22	A	A	A	A	G	A	C	A	A	T	T	A	A	T	T	A	T	T	393
pFS34	C	A	A	T	T	G	A	A	C	T	T	G	A	T	A	T	A	T	140
G	1	1	2	1	8	7	3	2	1	2	1	2	0	1	4	5	3	4	
A	19	20	6	4	5	9	10	20	6	3	5	19	19	7	6	9	18	3	
T	4	5	18	20	9	5	7	3	19	19	20	3	6	18	11	8	5	17	
C	3	1	1	2	5	6	7	2	1	3	1	3	2	1	6	5	1	3	
ASE	A	A	T	T	-	-	-	A	T	T	T	A	A	T	-	-	A	T	

Figure 31. Frequency of nucleotide occurrence in the putative ASE (anaerobic sense element) of all anaerobiosis-inducibble promoters. The speculated ASE of 14 known anaerobiosis-inducible genes and 4 unidentified promoters in this study were aligned and the frequency of occupancy for each position tabulated. Some of the anaerobiosis-inducible promoters have more than one putative ASE. The deduced consensus sequence for ASE is shown below the analysis. The number given after each nucleotide sequence is the position of the last nucleotide indicated in Figure 29.

II. Final discussions

A. Summary of the properties of the sequenced and characterized promoters cloned in this study.

Several anaerobiosis-inducible promoters have been cloned in this study. However only those with obvious anaerobiosis-induction were studied further.

For E. coli, I have cloned the promoter of glpT and demonstrated that the transcriptional expression of glpT is regulated by anaerobiosis. Its anaerobic expression was repressed by nitrate and glucose, while its aerobic expression was stimulated by the substrate a-glycerol-3-phosphate. The anaerobic expression of glpT was decreased by fnr mutation. Interestingly, the regulation of glpT was identical to that of glpA. glpA is transcribed divergently from glpT (Ehrmann et al., 1987) and the expression of glpA is also regulated by anaerobiosis (Kuritzkes et al., 1984). A putative FNR binding site in the upstream region of the transcriptional startsite of glpA has been suggested by Eiglmeier et al. (1989). The FNR binding site for glpT suggested in this study was the same nucleotide region suggested for glpA. Thus, we speculate that the FNR binding site can promote anaerobic expression in both orientation in the presence of CAP binding. (Figure 25, p138)

For S. typhimurium, I have cloned and sequenced

Table 21. Summary of the properties of plasmids pFE29, pHSK1, pHSK8, pFS22, and pFS34, which contains anaerobiosis-inducible promotors.

Plasmid	Gene fusion	Fragment size of insert (kb)	Anaerobic[a] induction ratio	*fnr*[b]	nitrate[c]	others
Escherichia coli promoter clones						
pFE29	*glpT-lac*	1.6	6	-	-	Glucose (-) α-G3P (+)
pFE32 (subcloned from pFE29)	*glpT-lac*	1.3	6	ND	0	Glucose (-) α-G3P (+)
pFE33 (subcloned from pFE29)	*glpA-lac*	0.72	6	ND	0	Glucose (-) α-G3P (+)
Salmonella typhimurium promoter clones						
pHSK1	X-*cat*[d]	7.1	40	ND	ND	
pHSK11 (subcloned from pHSK1)	X-*cat*	1.3	37	ND	ND	
pHSK8	X-*cat*	3.6	11	ND	ND	
pFSH8 (subcloned from pHSK8)	X-*lac*	1.98	11	-	+	Glucose (0)
pFS22	X-*lac*	0.49	20	0	+	Glucose (0)
pFS34	X-*lac*	0.55	8	0	0	Glucose (0)

a. Anaerobic induction ratio is defined as anaerobic activity/aerobic activity.

b. 0, activity no change in *fnr*(*oxrA*) mutation background; -, decreased activity in *fnr*(*oxrA*) mutation background; ND, not determined.

c. +, positive regulation, activity increase; -, negative regulation, activity decreased; 0, no activity change in the presence of exogenously added substrates. α-G3P is α-glycerol-3-phosphate.

d. X means unidentified gene.

four anaerobiosis-inducible promoter-containing DNA fragments. Two of them (contained by pHSK8 and pFS22) were also induced by nitrate under anaerobic condition. One of them (contained by pHSK8) was demonstrated to be fnr(oxrA)-dependent.

The properties of the studied promoter-containing DNA fragments were summarized in Table 21.

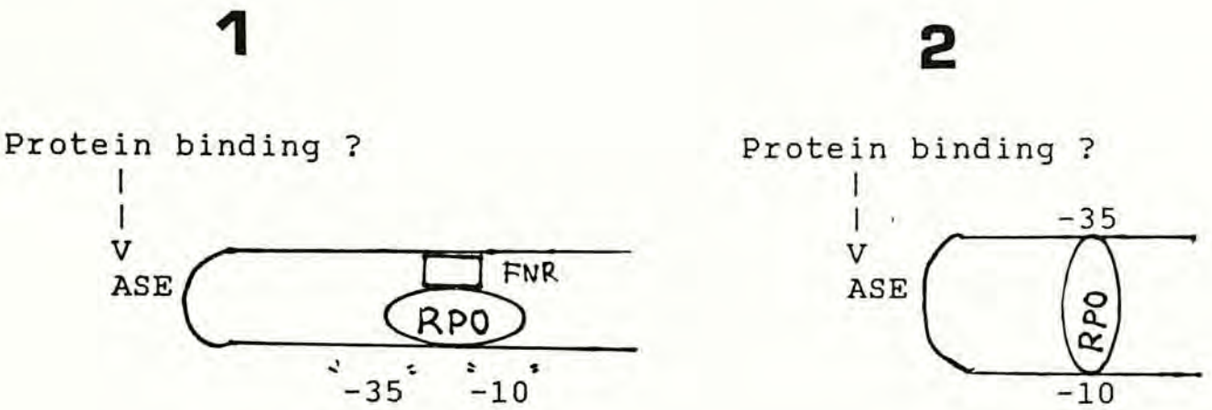
As regards to regulation, glpT and the promoter contained by pHSK8 have a putative FNR binding site and are fnr-dependent. However, pFS22 which is not fnr-dependent also contained a putative FNR binding site with the sequence 5'-GGCTAAAATGATATATATCAATGTAAAA-3. This site is 82% in homology with suggested consensus FNR binding site with half site well maintained. So, what is the function of this site if it was not for FNR binding is worth investigated. Similarly, the fragment carried by pFS34 also contained a putative FNR binding site with 75% homology and a half site maintained. Besides the putative FNR binding site, the fragment carried by pFS34 also contained conserved "-10" TATGAT and conserved "-35" TTGAAA but separated by 21 nucleotides. Aoyama and Takanami (1988) has shown that for a promoter carrying consensus sequence "-10" and "-35" which are separated by 19 or 20 nucleotides has no activity at relax state. But the activity increases when the superhelicity of the DNA increases. DNA conformation has been suggested to be involved in anaerobiosis regulation (Yamamoto and Droffner, 1985). This may be the case for the anaerobiosis regulation of the promoter carried by pFS34.

From homology search among the 5' noncoding DNA sequence of 14 known anaerobiosis-inducible genes (Figure 30) and the four unidentified promoters in this study, a consensus sequence called "anaerobiosis sense element" (ASE) with sequence 5'-AATT---ATTTAAT--AT-3' was proposed. The ASE is a AT rich region which may facilitate the bending of DNA for loop formation during activation by activators. Based on the fact that there are fnr-dependent and fnr-independent genes. Two models were proposed for the anaerobiosis-induction of these two set of genes (Figure 32).

ANAEROBIC

<u>fnr</u>-dependent genes

<u>fnr</u>-independent genes



supercoiling of DNA and bent DNA facilitate the binding of RNA polymerase (RPO) to "-10" and "-35" with long spacer length

1. Under anaerobic condition, FNR-Fe^{3+} senses the redox state and changes to FNR-Fe^{2+} which then binds to the FNR binding site. The binding of FNR facilitates the binding of RNA polymerase for transcription initiation with the bending at the ASE region.

2. Under anaerobic condition, negative supercoiling of the DNA and bending of DNA facilitate the binding of RNA polymerase for transcription initiation.

Figure 32. Schematic models for the anaerobic induction of <u>fnr</u>-dependent and <u>fnr</u>-independent genes.

B. Further studies

Many uncertainty still remains about the cloned promoters. Only one of the promoters was identified to be that of *glpT* of *E. coli*. Other promoters were not identified yet. The following experiments are suggested for further investigations:

1. By means of Northern blotting, we may be able to demonstrate that the DNA fragment cloned really has a RNA transcript *in vivo*.
2. Using S1 nuclease mapping or primer extension techniques to locate the transcriptional startpoint.
3. Extensive deletions and site directed mutagenesis should be used to define the functional domains in the promoter region.
4. One can locate map location of the cloned fragment in chromosome by introducing markers into the fragments such as mini-Mu, Tn5 or some cassette for later reciprocal homologous recombination into the chromosome via vectors.
5. The effect of other regulatory mutations such as *gyrA*, *oxrC*, *narL* etc. should be tested for these promoters.
6. Whether the FNR-binding site is a bacterial-enhancer-like element should be tested. Change the orientation and distance of this site from some well studied *fnr*-dependent promoters such as the *nar* promoter would give us some information.

REFERENCES

Aliabadi, Z., F. Warren, S. MYA, and J.W. Foster. 1986. Oxygen-regulated stimulons of Salmonella typhimurium identified by Mu d(Ap lac) operon fusions. J. Bacteriol. 165: 780-786.

Aoyama, T., and M. Takanami. 1988. Supercoiling response of E. coli promoters with different spacer lengths. Biochim. Biophys. Acta 949:311-317.

Auger, E.A., and G.N. Bennett. 1989. Regulation of lysine decarboxylase activity in Escherichia coli K-12. Arch Microbiol. 151: 466-468.

Ausubel, F.. 1984. Regulation of nitrogen fixation genes. Cell 37:5-6.

Axley, M.J., and T.C. Stadtman. 1988. Anaerobic induction of Escherichia coli formate dehydrogenase (hydrogenase-linked) is enhanced by gyrase inactivation. Proc. Natl. Acad. Sci. USA 85: 1023-1027.

Bachmann, B.. 1983. Linkage map of Escherichia coli K12, edition 7. Microbiol. Rev. 47:180-230.

Bally, V., M. Foglino, M. Bruschi, M. Murgier, and A. Lazdunski. 1986. Nucleotide sequence of the promoter and amino-terminal encoding region of the Escherichia coli pepN gene. Eur. J. Biochem. 155:565-569.

Barrett, E.L., H.S. Kwan, and J. Macy. 1984. Anaerobiosis, formate, nitrate, and pyrA are involved in the regulation of formate hydrogenlyase in Salmonella typhimurium. J. Bacteriol. 158:972-977.

Bell, A.I., K.L. Gaston, J.A. Cole, and S.J.W. Busby. 1989. Cloning of binding sequences for the Escherichia coli transcription activators FNR and CRP: location of bases involved in discrimaination between FNR and CRP. Nucl. Acids Res. 17: 3865-3874.

Bell, P.J., S.C. Andrews, M.N. Sivak, and J.R. Guest. 1989. Nucleotide sequence of the FNR-regulated furmarase gene (fumB) of Escherichia coli K-12. J. Bacteriol. 171: 3494-3503.

Bhriain, N.N., C.J. Dorman, and C.F. Higgins. 1989. An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. 3: 933-942.

Bilous, P.T., and J.H. Weiner. 1985. Dimethyl sulfoxide reductase activity by anaerobically grown Escherichia

coli HB101. J. Bacteriol. 162:1151-1155.

Bilous, P.T., S.T. Cole, W.F. Anderson, and J.H. Weiner. 1988. Nucleotide sequence of the dmsABC operon encoding the anaerobic dimethylsulphoxide reductase of Escherichia coli HB101. Mol. Microbiol. 2: 1-11.

Branlant, G., and C. Branlant. 1985. Nucleotide sequence of the Escherichia coli gap gene. Eur. J. Biochem. 150:61-66.

Brikmann, A., R.G. Sawers, and A. Böck. 1987. Involvement of the ntrA gene product in the anaerobic metabolism of Escherichia coli. Mol. Gen. Genet. 210: 535-542.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7:1513-1525.

Birnboim, H.C.. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. In Meth. Enzymol. 100:243-254.

Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy. 1989. Nitrate reductase of Escherichia coli: completion of the nucleotide sequence of the nar operon and reassessment of the role of the a and ß subunits in iron binding and electron transfer. Mol. Gen. Genet. 218: 249-256.

Bolivar, F., R.L. Rodriguez, M.C. Betlach, and H.W. Boyer. 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene 2:75-94.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brosius, J.. 1984. Plasmid vectors for the selection of promoters. Gene 27: 151-160.

Chan, Y.S. 1987. Anaerobic regulatory mutations in Salmonella typhimurium. Master of Philosophy Thesis. The Chinese University of Hong Kong.

Chen, E.Y., and P.H. Seeburg. 1985. Supercoiling sequencing: A fast and simple method for sequencing plasmid DNA. DNA 4:165-170.

Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M.C. Pascal. 1981. Operon fusions in the nitrate reductase operon and study of the control gene nirR in Escherichia coli. Mol. Gen. Genet. 182: 477-479.

Clark, D.P.. 1984. The number of anaerobically regulated genes in Escherichia coli. FEMS Microbiol. Lett. 24: 251-254.

Clark, M., and E.L. Barrett. 1987. The phs gene and hydrogen sulfide production by Salmonella typhimurium. J. Bacteriol. 169:2391-2397.

Cole, S.T., K. Eiglmeier, S. Ahmed, N. Honore, L. Elmes, W.F. Anderson, and J.H. Weiner. 1988. Nucleotide sequence and gene-polypeptide relationships of the glpABC operon encoding the anaerobic sn-glycerol-3-phosphate dehydrogenase of Escherichia coli K12. J. Bacteriol. 170: 2448-2456.

Cole, S.T.. 1982. Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of Escherichia coli. Eur. J. Biochem. 122: 479-482.

Cotter, P.A., and R.P. Gunsalus. 1989. Oxygen, nitrate, and molybdenum regulation of dmsABC gene expression in Escherichia coli. J. Bacteriol. 171: 3817-3823.

Davis, R.W., D. Botstein, J.R. Roth. 1980. In Advanced bacterial genetics. A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbo, N.Y.

Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of Escherichia coli by high voltage electroporation. Nucl. Acids Res. 16:6127-6145.

Ehrmann, M., W. Boos, E. Ormseth, H. Schwizer, and T.J. Larson. 1987. Divergent transcription of the sn-glycerol-3-phosphate active transport (glpT) and anaerobic sn-glycerol-3-phosphate dehydrogenase (glpA glpB glpC) genes of Escherichia coli K-12. J. Bacteriol. 169: 526-532.

Eiglmeier, K., W. Boos, and S.T. Cole. 1987. Nucleotide sequence and transcriptional startpoint of the glpT gene of Escherichia coli: extensive sequence homology of the glycero-3-phosphate transport protein with components of the hexose-6-phosphate transport system. Mol. Microbiol. 1: 251-258.

Eiglmeier, K., N. Honore, S. Iuchi, E.C.C. Lin, and S.T. Cole. 1989. Molecular genetic anaylsis of FNR-dependent promoters. Mol. Microbiol. 3: 869-878.

Eshoo, M. W.. 1988. lac Fusion Analsis of the bet Genes of Escherichia coli: Regulaton by Osmolarity, Temperature, Oxygen, Choline, and Glycine Betaine. J. Bacteriol. 170: 5208-5215.

Ely, B., R.M. Weppleman, H.C. Massey Jr., and P.E. Hartman. 1974. Some improved methods in P22 transduction. Genetics 76:625-631.

Fimmel, A.L., and B.A. Haddock. 1979. Use of chlC-lac fusions to determine regulation of gene chl in Escherichia coli. J. Bacteriol. 138: 726-730.

Fisher, L.M.. 1984. DNA supercoiling and gene expression. Nature (London) 307:686-687.

Foglino, M., S. Gharbi, and A. Lazdunski. 1986. Nucleotide sequence of the pepN gene encoding aminopeptidase N of Escherichia coli. Gene 49: 303-309.

Garciarrubio, A.A., and A.A. Covarrubias. 1987. Promoter selection by a bacterial enhancer-like activator element (BELE) in Escherichia coli. Gene 54: 275-280.

Gibson, M.M. , E.M. Ellis, K.A. Graeme-Cook, and C.F. Higgins. 1987. OmpR and EnvZ are pleiotropic regulatory proteins: Positive regulation of the tripeptide permase (tppB) of Salmonella typhimurium. Mol. Gen. Genet. 207: 120-129.

Goodlove, P.E., R.R. Cunningham, J. Parker, and D.P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of Escherichia coli. Gene 85: 209-214.

Griffiths, L., and J.A. Cole. 1987. Lack of redox control of the anaerobically-induced nirB+ gene of Escherichia coli K-12. Arch. Microbiol. 147: 364-369.

Gunsalus, R.P., L.V. Kalman, and R.R. Stewart. 1989. Nucleotide sequence of the narL gene that is involved in global regulation of nitrate controlled respiratory genes of Escherichia coli. Nucl. Acids Res. 17: 1965-1975.

Hallenbeck, P.C., M.A. Clark, and E.L. Barrett. 1989. Characterization of anaerobic sulfide reduction by Salmonella typhimurium and purification of the anaerobically induced sulfite reductase. J. Bacteriol. 171: 3008-3015.

Hanahan, D.. 1985. Techniques for transformation of E. coli, p109-135. In D.M. Glover (ed.), DNA cloning vol.1, a practical approach. IRL Press, Oxford, Washington DC.

Hanish, J., and M. McClelland. 1988. Activity of DNA modification and restriction enzymes in KGB, a potassium glutamate buffer. Gene Anal. Technol. 5: 105-107.

Harley, C.B., and R.P. Reynolds. 1987. Analysis of

Escherichia coli promotor sequences. Nucl. Acids Res. 15: 2343-2361.

Hawley, D.K., and W.R. McClure. 1983. Compilation and analysis of Escherichia coli promoter DNA sequences. Nucl. Acids Res. 11:2237-2249.

Hellinga, H.W., and P.R. Evans. 1985. nucleotide sequence and high-level expression of the major Escherichia coli phosphofructokinase. Eur. J. Biochem. 149:363-373.

Henikoff, S.. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28: 351-359.

Hirschman, J. P.K. Wong, K. Sei, J. Keener, and S. Kustu. 1985. Products of nitrogen regulatory genes ntrA and ntrC of enteric bacteria activate glnA transcription in vitro: evidence that the ntrA product is a σ factor. Proc. Natl. Acad. Sci. USA 82:7525-7529.

Holley, E.A. , M. P. Spector, and J. W. Foster. 1985. Regulation of NAD biosynthesis in Salmonella typhimurium: Expression of nad-lac gene fusions and identification of a nad regulatory locus. J. Gen. Microbiol. 131: 2759-2770.

Hughes, D.E., J. E. Curtis, C. Khosla, and J.E. Bailey. 1989. A New oxygen-regulated promoter for the expression of proteins in Escherichia coli. Biotechnol. 7: 1026-1028.

Iuchi, S., D.R. Kuritzkes, and E.C.C. Lin. 1985. Escherichia coli mutant with altered respiratory control of the frd operon. J. Bacteriol. 161:1415-1421.

Iuchi, S., and E.C.C. Lin. 1987. The narL gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine N-oxide reductase operons in Escherichia coli. Proc. Natl. Acad. Sci. USA 84: 3901-3905.

Iuchi, S., and E.C.C. Lin. 1988. arcA(dye), a global regulatory gene in Escherichia coli mediating repression enxymes in aerobic pathways. Proc. Natl. Acad. Sci. USA 85: 1888-1892.

Iuchi, S., R. Kuritzkes, and E.C.C. Lin. 1986. Three Classes of Escherichia coli Mutants selected for aerobic expression of fumarate reductase. J. Bacteriol. 168: 1415-1421.

Jamieson, D.J., and C.F. Higgins. 1984. Anaerobic and leucine-dependent expression of a peptide transport gene in Salmonella typhimurium. J. Bacteriol. 160: 131-136.

Jamieson, D.J., and C.F. Higgins. 1985. Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in Salmonella typhimurium. J. Bacteriol. 168: 389-397.

Jamieson, D. J., R. G. Sawers, P. A. Rugman, D. H. Boxer, and C. F. Higgins. 1986. Effects of anaerobic regulatory mutations and catabolite repression on regulation of hydrogen metabolism and hydrogenase isoenzyme composition in Salmonella typhimutium. J. Bacteriol. 168: 405-411.

Jayaraman, P. S., T. C. Peakman, S. J. W. Busby, R. V. Quincey, and J. A. Cole. 1987. Location and sequence of the promoter of the gene for the NADH-dependent nitrite reductase of Escherichia coli and its regulation by oxygen, the Fnr protein and nitrite. J. Mol. Biol. 196: 781-788.

Jayaraman, P. S., K. L. Gaston, J.A. Cole, and J.W. Busby. 1988. The nirB promoter of Escherichia coli: location fo nucleotide sequences essential for regulation by oxygen, the FNR protein and nitrite. Mol. Microbiol. 2: 527-530.

Jayaraman, P. S., J. A. Cole, and S. J. W, Busby. 1989. Mutational analysis of the nucleotide sequence at the FNR-dependent nirB promoter in Escherichia coli. Nucl. Acids Res. 17: 135-145.

Jennings, M.P., and I.R. Beacham. 1990. Analysis of the Escherichia coli gene encoding L-asparaginase II, ansB, and its regulation by cyclic AMP receptor and FNR proteins. J. Bacterial. 172:1491-1498.

Jerlstrm, P.G., J. Lui, and I.R. Beacham. 1987. Regulation of Escherichia coli L-asparaginase II and L-asparatase by the fnr gene-product. FEMS Microbiol. Lett. 47:127-130.

Jeter, R.M., and J.R. Roth. 1987. Cobalamin (Vitamin B12) biosynthetic genes of Salmonella typhimurium. J. Bacteriol. 169: 3189-3198.

Jones, H.M., and R.P. Gunsalus. 1985. Transcription of the Escherichia coli fumarate reductase genes (frdABCD) and their coordinate regulation by oxygen, nitrate, and fumarate. J. Bacteriol. 164: 1100-1109.

Jones, H.M., and R.P. Gunsalus. 1987. Regulation of Escherichia coli fumarate reductase (frdABCD) operon expression by respiratory electron acceptors and the fnr gene product. J. Bacteriol. 169: 3340-3349.

Kalman, L.V., and R.P. Gunsalus. 1988. The frdR gene of Escherichia coli globally regulates several operons involved in anaerobic growth in response to nitrate. J. Bacteriol. 170: 623-629.

Kalman, L., and R.P. Gunsalus. 1989. Identification of a second gene involved in global regulation fumarate reductase and other nitrate-controled genes for anaerobic respiration in Escherichia coli. J. Bacteriol. 171: 3810-3816.

Koop, A.H., M.E. Hartley, and S. Bourgeois. 1987. A low-copy-number vector utilizing ß-galactosidase for the analysis of gene control elements. Gene. 52:245-256.

Kukral, A. M., K. L. Strauch, R. A. Maurer, and C. G. Miller. 1987. Genetic Analysis in Salmonella typhimurium with a Small Collection of Randomly Spaced Insertions of Transposon Tn10^16^17. J. Bacteriol. 169: 1787-1793.

Kuritzkes, D. R., X.-Y. Zhang, and E.C.C. Lin. 1984. Use of φ(glp-lac) in the studies of respiratory regulation of the Escherichia coli anaerobic sn-glycerol-3-phosphate dehydrogenase genes (glpAB). J. Bacteriol. 157: 591-598.

Kwan, H.S., and E.L. Barrett. 1983. Roles for menaquinone and the two trimethylamine oxide (TMAO) reductase in TMAO respiration in Salmonella typhimurium Mud(Ap^r lac) insertion mutations in men and tor. J. Bacteriol. 155:1147-1155.

Kwan, H.S., H.W. Chui, and K.K. Wong. 1988. ack::Mud1-8(Ap^r lac) operon fusions of Salmonella typhimurium LT2. Mol. Gen. Genet. 211:183-185.

Kwan, H.S., and K.K. Wong. 1986. A general method isolation of Mu d1-8(Ap^r lac) operon fusions in Salmonella typhimurium LT2 from Tn10 insertion strains: chlC::Mu d1-8. Mol. Gen. Genet. 205:221-224.

Lambden, P.R., and J.R. Guest. 1976. Mutants of Escherichia coli K12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. 97:145-160.

Li, S.F., and J.A. DeMoss. 1987. Promoter region of the nar operon of Escherichia coli: nucleotide sequence and transcription initiation signals. J. Bacteriol. 169:4614-4620.

Li, S.F., and J.A. DeMoss. 1988. Location of sequences in the nar promoter of Escherichia coli required for regulation by Fnr and NarL. J. Biol. Chem. 263: 13700-13705.

Lorowitz, W., and D. Clark. 1982. Escherichia coli mutants

with a temperature-sensitive alcohol dehydrogenase. J. Bacteriol. 152:935-938.

Matsuyaam, A., H. Yamamoto, and E. Nakano. 1989. Cloning, expression , and nucleotide sequence of Escherchia coli K-12 ackA gene. J. Bacteriol 171: 577-580.

Messing, J. 1983. New M13 vectors for cloning. Meth. Enzymol. 101:20-78.

Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mizusawa, S., S. Nishimura, and F. Seela. 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7 deazaguanosine triphosphate in place of dGTP. Nucl. Acids Res. 14:1319-1324.

Newman, B.M., and J.A. Cole. 1978. The chromosome location and pleiotropic effects of mutations of the nirA$^+$ gene of Escherichia coli K12: The essential role of the nirA$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. 106:1-12.

Nellemann, L.J., F. Holm, T. Altung, and F.G. Hansen. 1989. Cloning and charactrerization of the Escherichia coli phosphoglycerate kinase (pgk) gene. Gene 77: 185-191.

Nohno, T., S. Noji, S. Taniguchi, and T. Saito. 1989. The narX and narL genes encoding the nitrate-sensing regulators of Escherichia coli are homologous to a family of prokaryotic two-component regulatory genes. Nucl. Acids Res. 17: 2947-2957.

Noji, S., T. Nohno, T. Saito, and S. Taniguchi. 1989. The narK gene product participates in nitrate transport induced in Escherichia coli nitrate-respiring cells. FEBS Lett. 252:139-143.

O'Farrel, P.Z., H.M. Goofmsn, and P.H. O'Farrel. 1977. High resolution two-dimensional eolectrophoresis of basic as well as acidic proteins. Cell 12:1133-1142.

Pascal. M-C., J-F. Burini, and M. Chippaux. 1984. Regulation of the trimethylamine N-oxide (TMAO) reductase in Escherichia coli: Analysis of tor::Mud1 operon fusion. Mol. Gen. Genet. 195: 351-355.

Pascal, M-C., V. Bonnefoy, M Fons, and M Chippaux. 1986. Use of gene fusions to study the expression of fnr, the regulatory gene of anaerobic electron transfer in Escherichia coli. FEMS Microbiol. Lett. 36: 35-39.

Paveglio, M.T., J.S. Tang, R.E. Unger, and E.L. Barrett. 1988. Formate-nitrate respiration in Salmonella

typhimurium: Studies of two rha-linked fdn genes. J. Bacteriol. 170: 213-217.

Pecher, A., H. P. Blaschkowski, K. Knappe, and A. Böck. 1982. Expression of pyruvate formate-lyase of Escherichia coli from the cloned structural gene. Arch. Microbiol. 132: 365-371.

Pecher, A., F. Zinoni, C. Jatisatienr, R. Wirth, H. Hennecke, and A. Böck. 1983. On the redox control of synthesis of anaerobically induced enzymes in enterobacteriaceae. Arch. Mircobiol. 136: 131-136.

Privalle, C.T., W.F. Beyer, and I. Fridovich. 1989. Anaerobic induction of proMn-superoxide dismutase in Escherichia coli. J. Biol. Chem. 264: 2758-2763.

Reams, S.G., J.W. Winkelman, and D.P. Clark. 1987. Anaerobic induction of genes involved in the biosynthesis of amino acids and thiamine, in Escherichia coli. FEMS Microbiol Lett. 42: 231-237.

Reichelt, J.L., and H.W. Doelle. 1971. The influence of dissolved oxygen concentration on phosphofructokinase and the glucose metabolism of Escherichia coli K-12. Antonie Van Leeuwenhoek J. Microbiol. Seriol. 37:497-506.

Rodel, W., W. Plaga, R. Frank, and J. Knappe. 1988. Primary structures of Escherichia coli pyruvate formate-lyase and pyruvate-formate-lyase-activating enzyme deduced from the DNA nucleotide sequences. Eur. J. Biochem. 177: 153-158.

Ryu, J., and R.J. Hartin. 1990. Quick transformation in Salmonella typhimurium LT2. Biotechniques 8: 43-45.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Sanderson, K.E., and J.R. Roth. 1988. Linkage Map of Salmonella typhimurium, edition VII. Microbiol. Rev. 52: 485-532.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Sawers, R. G., E. Zehelein, and A. Böck. 1988. Two dimensional gel electrophoretic analysis of Escherichia coli proteins: influence of various anaerobic growth conditions and the fnr gene product on cellular protein composition. Arch Microbiol. 149: 240-244.

Sawers, G., and A. Böck. 1988. Anaerobic Regulation of Pyruvate Formate-Lyase from Escherichia coli K-12. J. Bacteriol. 170: 5330-5336.

Sawers, G., and A. Böck. 1989. Novel transcriptional control of the pyruvate formate-lyase gene: Upstream regulatory sequences and multiple promoters regulated anaerobic expression. J. Bacteriol. 171: 2485-2498.

Sawers, R.G., S.P. Ballantine, and D.H. Boxer. 1985. Differential expression of hydrogenase isoenxymes in Escherichia coli K-12: evidence for a third isoenzyme. J. Bacteriol. 164: 1324-1331.

Shaw, W.V.. 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Meth. Enzymol. 43:737-755.

Shaw, D.J., and J.R. Guest. 1981. Molecular cloning of the fnr gene of Escherichia coli K12. Mol. Gen. Genet. 181: 95-100.

Shaw, D.J., and J.R. Guest. 1982. Amplification and product identification of the fnr gene of Escherichia coli. J. Gen. Microbiol. 128: 2221-2228.

Shaw, D.J., D.W. Rice, J.R. Guest, and J. Miller. 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in Escherichia coli. J. Mol. Biol. 166: 241-247.

Showe, M.K. , and J.A. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in Escherichia coli. J. Bacteriol. 95:1305-1313.

Smith, M.W., and F.C. Neidhardt. 1983a. Proteins induced by anaerobiosis in Escherichia coli. J. Bacteriol. 154: 336-343.

Smith, M.W., and F.C. Neidhardt. 1983b. Proteins induced by aerobiosis in Escherichia coli. J. Bacteriol. 154:344-350.

Spector, M.P., Z. Aliabadi, T. Gonzalez, and J.W. Foster. 1986. Global control in Salmonella typhimurium: Two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. J. Bacteriol. 168: 420-424.

Spiro, S., and J.R. Guest. 1987a. Activation of the lac operon of Escherichia coli by a mutant FNR protein. Mol. Microbiol. 1:53-58.

Spiro, S., and J.R. Guest. 1987b. Regulation and over-

expression of the fnr gene of Escherichia coli. J. Gen. Microbiol. 133: 3279-3288.

Stewart, V., and B.L. Berg. 1988. Influence of nar (nitrate reductase) genes on nitrate inhibition of formate-hydrogen lyase and fumarate reductase gene expression in Escherichia coli K-12. J. Bacteriol. 170: 4437-4444.

Stewart, V., and J. Parales Jr.. 1988. Identification and expression of genes narL and narX of the nar (nitrate reductase) locus in Escherichia coli K-12. J. Bacteriol. 170: 1589-1597.

Stewart V., J. Parales Jr., and S. M. Merkel. 1989. Structure of Genes narL and narX of the nar (Nitrate Reductase) Locus in Escherichia coli K-12. J. Bacteriol. 171: 2229-2234.

Stewart, V. 1982. Requirement of fnr and narL functions for nitrate reductase expression in Escherichia coli K-12. J. Bacteriol. 151: 1320-1325.

Stewart, V.. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. 52: 190-232.

Strauch, K.L., J.B. Lenk, B.L. Gamble, and C.G. Miller. 1985. Oxygen regulation in Salmonella typhimurium. J. Bacteriol. 161: 673-680.

Studnicka, G. M.. 1987. Nucleotide sequence homologies in control regions of prokaryotic genomes. Gene 58: 45-57.

Tang, J.S., and Barrett. 1986. Regulation of anaerobic metabolism in Salmonella typhimurium involves a genetic locus distinct from oxrA. In Abstracts of the Annual Meeting of the American Society for Microbiology 1986. Washington, D.C. USA.

Thomas, A.D., H.W. Doelle, A.W. Westwood, and G.L. Gordon. 1972. Effect of oxygen on several enzymes involved in aerobic and anaerobic utilization of glucose in Escherichia coli. J. Bacteriol. 112:1099-1105.

Tsai, S.P., R.J. Hartin, and J. Ryu. 1989. Transformation in restriction-deficient Salmonella typhimurium LT2. J. Gen. Microbiol. 135:2561-2567.

Unden, G., and R. Guest. 1984. Cyclic AMP and anaerobic gene expression in Escherichia coli. FEBS Lett. 170: 321-325.

Unden, G.. 1988. Differential roles for menaquinone and dimethylmenaquinone in anaerobic electron transport of E. coli and their fnr-independent expression. Arch. Microbiol. 150: 499-503.

Volkert, M. R., L. I. Hajec, and D. C. Nguyen. 1989. Induction of the Alkylation-Inducible aidB Gene of Escherichia coli by Anaerobiosis. J. Bacteriol. 171: 1196-1198.

Winkelman, J.W., and D.P. Clark. 1986. Anaerobically induced genes of Escherichia coli. J. Bacteriol. 167: 362-367.

Wong, P.K., and E.L. Barrett. 1983. Aerobic and anaerobic alcohol dehydrogenases in Escherichia coli. FEMS Microbiol. Lett. 22:143-148.

Wong, K.K., and H.S. Kwan. 1988. A novel mutation causing the deficiency in lactate production of Salmonella typhimurium during mixed acid fermentation, p290-300. In S.T. Chang, K.Y. Chan, and N.Y.S. Woo (ed.), Recent Advances in Applied Biology and Biotechnology. The Chineses University Press, Hong Kong.

Wong, K.K., K. L. Suen, and H.S. Kwan. 1989. Transcription of pfl is regulated by anaerobiosis, catabolite repression, pyruvate, and oxrA: pfl::Mu dA operon fusions of Salmonella typhimurim. J. Bacteriol. 171: 4900-4905.

Woods, S.A., and J.R. Guest. 1987. Differential roles of the Escherichia coli fumarases and fnr-dependent expression of fumarase B and asparatase. FEMS Microbiol. Lett. 48: 219-224.

Wu, L.F., and M.A. Mandrand-Berthelot. 1987. Regulation of the fdhF gene encoding the selenopolypeptide for benzyl viologen-linked formate dehydrogenase in Escherichia coli. Mol. Gen. Genet. 209: 129-134.

Yamamoto, N, and M.L. Droffner. 1985. Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe Salmonella typhimurium. Proc. Natl. Acad. Sci. USA 82: 2077-2081.

Yanisch-Perron, C., J. Vieira, and J. Mesing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene 33: 103-119.

Yerkes, J.H., L.P. Casson, A.K. Honkanen, and G.C. Walker. 1984. Anaerobiossis induces expression of ant, a new Escherichia coli locus with a role in anaerobic electron transport. J. Bacteriol. 158: 180-186.

Zinoni, F., A. Beier, A. Pecher, R. Wirth, and A. Böck. 1984. Regulation of the synthesis of hydrogenase (formate hydrogen-lyase linked) of E. coli. Arch. Microbiol. 139: 299-304.

Zinoni, F., A. Birkmann, T.C. Stadtman, and A. Böck. 1986. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli. Proc. Natl. Acad. Sci. USA 83:4650-4654.